Regulation of RNA polymerase III transcription during cardiomyocyte hypertrophy

Sarah Jayne Goodfellow

Thesis submitted for the Degree of Doctor of Philosophy

January 2005

UNIVERSITY
of
GLASGOW

Division of Biochemistry & Molecular Biology

Institute of Biomedical & Life Sciences

University of Glasgow

Acknowledgements

I am extremely grateful to my supervisors Dr P Scott and Professor R White for all their valuable advice and encouragement throughout my time in the laboratory, and whilst writing this thesis.

I would also like to thank Dr Fiona Cairns for supplying and advising me on the use of recombinant adenoviruses, Ms Louise Derblay for her assistance with preparing cardiomyocyte cultures, Dr G May and Dr A Kondrashov (Division of Biochemistry & Molecular Biology, University of Glasgow) for their advice on the use of Bioinformatics tools, and all my other colleagues in the lab, particularly Emma Graham, for their friendship and support.

Finally, I thank my partner Paul and my family for their care and patience over the past three years.

This project was funded by The Welcome Trust.

Summary

In comparison to cell division, the mechanisms underlying cell growth are poorly understood. Progress towards understanding the latter requires systems in which cell growth can be studied independently from proliferation. Cardiomyocytes terminally differentiate and lose the capacity to divide shortly after birth in mammals. Enlargement of existing cardiomyocytes accounts for the increase in heart size that occurs during post-natal development. This increase in cell size, in the absence of proliferation, is known as hypertrophy. Cardiomyocyte hypertrophy also occurs in the adult heart in response to a range of physiological stimuli, allowing the heart to adjust its contractile capacity according to demand. However, if sustained, cardiomyocyte hypertrophy is associated with several serious cardiovascular disorders, and is an independent risk factor for heart disease.

One of the key hallmarks of hypertrophy is an increase in protein synthesis; however, the mechanisms responsible for this increased translation are not fully understood. RNA polymerase III (pol III) plays a vital role in protein synthesis by producing several components of the cellular biosynthetic machinery, including transfer (t)RNAs and 5S ribosomal (r)RNA. Thus, this project aimed to investigate whether pol III transcription is upregulated during hypertrophic growth, and if so, to elucidate the mechanisms responsible for such control.

Various stimuli induce hypertrophy in primary cultures of rat cardiomyocytes. Data presented in this thesis show that this hypertrophic growth accompanies a substantial increase in transcription by pol III, along with markedly elevated levels of various pol III products, including tRNAs and 5S rRNA. Furthermore, analysis of mouse ventricular tissue demonstrated that pol III transcription is also enhanced during hypertrophy in the myocardium *in situ*.

Several proteins are known to regulate transcription by pol III in proliferating cell lines. These include activators, such as c-Myc and extracellular signal-regulated kinase (ERK), and repressors, such as the retinoblastoma protein (RB).

In addition, these molecules have been implicated in the regulation of cardiomyocyte hypertrophy; therefore, their involvement in pol III transcriptional control was investigated in these terminally differentiated cells. This revealed that c-Myc and ERK, which are known to promote hypertrophic growth, can activate pol III transcription in cardiomyocytes. On the other hand, although normally associated with controlling the cell division cycle, RB has recently been implicated as a negative regulator of hypertrophy, and in the present study, RB was shown to attenuate transcription by pol III in the heart. The activation or inhibition of pol III transcription by these proteins is likely to contribute to their ability to induce or repress hypertrophic cell growth.

Therefore, proliferating and non-dividing terminally differentiated cells appear to use some common means to regulate pol III transcription, and hence cellular biosynthetic capacity. However, a novel pol III transcriptional control mechanism was also identified in this study, namely the hypertrophy-associated, ERK-mediated induction of the pivotal pol III-specific transcription factor Brf1. Further work is required to establish whether Brf1 induction contributes to increased pol III transcription during the growth of other terminally differentiated cell types, or whether this mechanism is unique to cardiomyocytes. Thus, numerous mechanisms contribute to the control of transcription by pol III in cardiomyocytes, suggesting that such regulation is a critical determinant of hypertrophic cell growth.

In summary, this thesis has identified a previously undescribed means by which hypertrophic stimuli could increase the protein synthetic capacity of cardiomyocytes, and has delineated the mechanisms responsible. This has important implications for understanding the molecular basis of pathological cardiomyocyte hypertrophy, and cell growth in general.

Contents

List of Figures

Chapter 4

Chapter 5

List of Tables

List of Abbreviations

4E-BP1	eIF4E-binding protein 1
ARPP P0	acidic ribosomal phosphoprotein P0
ANF	atrial natriuretic factor
arg	arginine
B	HA-Brf1 expressing adenovirus
bp	base pairs
β-gal	β-galactosidase
b/HLH/z	basic/helix-loop-helix/leucine zipper
BrdU	5-bromodeoxyuridine
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
BSA	bovine serum albumin
CAMEK	constitutively active MEK1
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DSE	distal sequence element
DTT	dithiolthreitol
eEF2	eukaryotic elongation factor 2

eIF2Bε	eukaryotic initiation factor 2Bε
eIF4E	eukaryotic initiation factor 4E
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
F	forward primer
FCS	foetal calf serum
G	GFP-expressing adenovirus
GFP	green fluorescent protein
HA	haemagglutinin
HA-Brf1	HA-tagged Brf1
HAT	histone acetyltransferase
HDAC	histone deacetylase
HPV	human papillomavirus
HS	horse serum
ICR	internal control region
IE	intermediate element
IIIB	TFIIIB
IIIC	TFIIIC
IVTs	*In vitro* transcription assays
JNKs	c-Jun N-terminal kinases
KO	mice lacking RB in the myocardium
leu	leucine
M199	medium 199
MAP	mitogen-activated protein
MEK	MAP kinase kinase

MEKK	MAP kinase kinase kinase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
Myc	c-Myc
MycER	c-Myc-oestrogen receptor fusion protein
OHT	4-hydroxytamoxifen
p	probability
PD	PD98059
PBS	phosphate-buffered saline
PE	phenylephrine
phos-	phosphorylated
PKC	protein kinase C
PI3K	phosphatidylinositol 3-kinase
pol I	RNA polymerase I
pol II	RNA polymerase II
pol III	RNA polymerase III
PSE	proximal sequence element
R	reverse primer
RB	retinoblastoma protein
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
S	DNA synthesis
S6Ks	ribosomal protein S6 kinases
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

ser	serine
SF	serum-free
SINEs	short interspersed elements
snRNA	small nuclear RNA
TAC	transverse aortic constriction
TAD	transactivation domain
TATA	TATA box
TBP	TATA box-binding protein
TOR	target of rapamycin
tg	transgenic
thr	threonine
tRNA	transfer RNA
WT	wild-type

CHAPTER 1
Introduction

1.1 Eukaryotic transcription and cell growth

Cellular functions, including growth, division, differentiation and death, are ultimately determined by the appropriate expression of specific genes. The first stage of gene expression is transcription, a process by which large, complex RNA polymerase enzymes synthesise RNA using specific DNA templates. In eukaryotic cells, the task of transcribing nuclear genes is shared by RNA polymerases I, II and III (White, 1998). Each of these polymerases is dedicated to the transcription of a different set of genes, known as class I, II or III genes, accordingly. RNA polymerase I (pol I) transcribes genes encoding the 45S ribosomal (r)RNA, which serves as a precursor for the 5.8S, 18S and 28S rRNAs (Grummt, 2003). This accounts for up to 60% of cellular RNA synthesis (Moss and Stefanovsky, 2002). RNA polymerase II (pol II) produces messenger (m)RNAs, which code for cellular proteins, and many small nuclear (sn)RNAs, which are involved in mRNA processing (White, 1998). RNA polymerase III (pol III) is responsible for 10 to 20% of all nuclear transcription (Moss and Stefanovsky, 2002), and synthesises a variety of small, untranslated RNAs with essential roles in metabolism, including transfer (t)RNAs and 5S rRNA (White, 2002). Tight regulation of these polymerases, and their respective transcription machineries, dictates gene expression patterns, and hence cell function. Although the proteins encoded by class II genes function in a diverse array of processes, the untranslated RNAs produced by pols I and III are primarily involved with

biosynthesis. This thesis is specifically concerned with pol III transcription and its relevance to cell growth.

Cell growth is fundamental to eukaryotic life. Although cell growth and division are often considered together, these processes are distinct, with growth resulting in an increase in cell mass, and division leading to increased cell number. Without cell growth, division could not be sustained, as cells would become progressively smaller. Thus, in general, cell growth is rate-limiting for cell division (Johnston et al, 1977; Nurse, 1975). In fact, several studies have shown that cells must reach a sufficient mass before they will duplicate their DNA and divide (Johnston et al, 1977; Killander and Zetterberg, 1965; Rønning et al, 1981; reviewed by Conlon and Raff, 1999; Fingar et al, 2002). Knowledge of the cell division cycle has increased enormously in the last three decades, following extensive studies with yeast models, then complementary investigations in mammalian cells. However, by comparison, the current understanding of cell growth control is fairly limited, despite its primary importance. Therefore, the focus of research is turning towards elucidating the mechanisms underlying cell growth.

Cell growth is largely due to protein accumulation, which usually occurs as a result of increased rates of protein synthesis (Baxter and Stanners, 1978). Ribosomes constitute the core of the protein synthetic machinery; consequently, ribosome content is a critical determinant of protein accumulation (Brandenburger et al, 2001; Camacho et al, 1990; Siehl et al, 1985; Zetterberg and Killander, 1965). The abundance of ribosomes within a cell depends on the

availability of rRNA (Liebhaber et al, 1978), which, as mentioned above, is produced by pols I and III. Furthermore, along with ribosomes, the accurate and efficient execution of translation requires many specialised, pol III-produced tRNA species, which, accordingly, also impinge upon cell growth rate (Francis and Rajbhandary, 1990). Moreover, pol III synthesises an array of additional RNAs that also function in various aspects of cellular biosynthesis, as discussed in the following sections. Therefore, regulating class III gene expression clearly has the potential to influence the capacity for cell growth.

Although cell division does not usually occur without prior growth, cell growth can occur independently of proliferation. For example, mutations that specifically block the cell division cycle do not always halt cell growth (Johnston et al, 1977; Neufeld et al, 1998; Weigmann et al, 1997). Furthermore, some terminally differentiated animal cells that have lost the capacity to divide (such as heart and skeletal myocytes and nerve cells), continue to grow during development and in adults in response to growth stimuli (Conlon and Raff, 1999). This type of cell growth is known as hypertrophy, and because it occurs in the absence of cell division, provides a useful means for studying mechanisms contributing directly to growth. The hypertrophic growth of cardiomyocytes has received considerable attention in recent years. These cardiac muscle cells, which constitute the bulk of heart mass, withdraw from the cell cycle in the neonate, but continue to grow during post-natal development in order to attain the appropriate adult heart size (Bugaisky et al, 1992; Hannan and Rothblum, 1995; Leu et al, 2001; Zak, 1974). Cardiomyocyte hypertrophy can also occur in the adult heart as an adaptive response to increased workloads and hormonal cues (Chien et al,

1991). However, aberrant cardiomyocyte growth is an independent risk factor for cardiovascular disease, thus prompting intensive investigation into the molecular mechanisms underlying this response (Harjai et al, 1999; Kannel, 1990; Levy et al, 1990; Vakili et al, 2001). Although this has led to the characterisation of initiating stimuli and intracellular signalling pathways involved, it remains unclear how these induce hypertrophy. This thesis explores the possibility that changes in pol III transcription contribute to this cell growth control. Before addressing this issue, it is important to appreciate the various biosynthetic functions of pol III-synthesised RNAs, the transcription machinery involved in their production, and how such transcription can be regulated. These aspects of class III gene expression are discussed in this introduction.

1.2 Class III genes

The genes transcribed by pol III encode a variety of RNAs, typically less than 400 nucleotides in length. These RNAs, and their functions, are listed in Table 1. The sections that follow discuss some of these transcripts in more detail.

Table 1: Summary of class III gene products and their functions*

Pol III Product	Known Functions
5S rRNA	Involved in protein synthesis as a component of ribosomes
tRNA	Involved in protein synthesis as a translational adaptor
U6 snRNA	Involved in mRNA splicing
7SL RNA	Involved in intracellular protein transport (component of signal recognition particle)
H1 RNA	Involved in tRNA processing (RNase P component)
MRP RNA	Involved in large rRNA splicing and mitochondrial RNA processing
7SK RNA	Involved in controlling transcriptional elongation by pol II
vault RNA	Component of cytoplasmic vault ribonucleoproteins
SINE transcripts	Not well defined. Potential role in cellular stress responses
VA RNAs	Involved in adenovirus translational control
EBER RNAs	Thought to be involved in Epstein-Barr virus translational control

*this information is reviewed in White, 2002.

1.2.1 5S rRNA

Along with approximately 80 proteins (Doudna and Rath, 2002), and the 5.8S, 18S and 28S rRNAs produced by pol I, the 120 nucleotide 5S rRNA is an essential component of ribosomes in every eukaryotic organism, and therefore is vital for protein synthesis (Wool, 1979). As with all class III genes, 5S is

transcribed in the nucleoplasm. However, following synthesis, 5S rRNA is transported to the nucleolus where it is processed and incorporated into the large ribosomal subunit, which specifically catalyses the formation of peptide bonds (Lafontaine and Tollervey, 2001). Eukaryotic genomes contain multiple 5S genes, ranging from 140 in the haploid genome of the yeast *Saccharomyces cerevisiae*, to more than 20 000 copies in *Xenopus laevis* (Brown, 1971; Elion and Warner, 1984). The majority of these *X. laevis* 5S genes are only expressed in the oocyte in order to sustain the rapid growth required during this developmental stage (Wolffe and Brown, 1988). Human cells are thought to contain 200 to 300 5S genes present in tandem arrays (International Human Genome Sequencing Consortium, 2001). In addition, 5S gene variants and untranscribed pseudogenes have been described in some species (White, 2002).

1.2.2 tRNAs

As with 5S rRNA, tRNAs play an essential role in mRNA translation. These molecules serve as adaptors, allowing the genetic information carried in a particular nucleotide sequence to be translated into the appropriate amino acid sequence by the ribosome. Following their initial synthesis, tRNA transcripts are processed, and in some cases covalently modified, resulting in mature tRNAs which range in length from 70 to 90 nucleotides, and adopt a highly structured L-shaped conformation (Creighton, 1997; Hopper and Phizicky, 2003). As indicated in Table 1, the class III gene product H1 participates in this tRNA processing as part of the RNase P endoribonuclease (Bartkiewicz et al, 1989; Lee and Engelke, 1989; Lee et al, 1991). There are approximately 50 to 100 distinct tRNA species within a eukaryotic cell (Sharp et al, 1984); in the haploid human

genome, these are encoded by around 500 genes (International Human Genome Sequencing Consortium, 2001). Although each of the expressed tRNA species has a similar, L-shaped structure, their unique nucleotide sequences allow functional specificity. For example, each tRNA will just recognise one particular amino acid and deliver this to the growing polypeptide chain only if matched to a complementary codon in the mRNA. Thus, tRNAs are vital for the fidelity of protein synthesis.

1.2.3 U6 snRNA

Following their initial synthesis by pol II, pre-mRNAs are extensively processed prior to translation. For example, 5' and 3' end modifications take place and, in addition, splicing is required to remove non-coding intron regions, thus producing a continuous coding sequence compatible with the translation machinery. Pre-mRNA splicing occurs in the nuclei of all eukaryotic cells and is performed by spliceosomes. Spliceosomes are abundant complexes, containing 5 separate snRNA species and many proteins (Hastings and Krainer, 2001). Four of these snRNAs are produced by pol II; however, the smallest (106 nucleotides), most highly conserved snRNA U6 is manufactured by pol III (Reddy et al, 1987). The high degree of U6 conservation between different organisms likely reflects the prominant role this particular RNA plays in directly catalysing the splicing reaction (Nilsen, 2000; Southeimer, 2001; Yean et al, 2000).

1.2.4 7SL RNA

The class III gene 7SL encodes the 300 nucleotide RNA component of the signal recognition particle (White, 2002). This signal recognition particle, which also

contains six polypeptides, is responsible for the appropriate targeting of ribosomes engaged in translation to the endoplasmic reticulum, thus delivering nascent polypeptide chains to this organelle, where they are modified, correctly folded and then further directed to their final destinations (Creighton, 1997).

1.2.5 SINEs

In higher eukaryotes, pol III transcribes several short interspersed elements or SINEs. In humans, the most predominant SINEs are the Alu genes, whereas in rodents the B1 and B2 SINE families are most abundant. B1 genes are highly homologous to Alu genes, and both appear to have evolved from the 7SL gene; however, B2 genes are rodent-specific, and are thought to have evolved from tRNA genes (White, 2002). These SINE families constitute a significant fraction of mammalian genomes: over a million copies of Alu are encoded by the haploid human genome (International Human Genome Sequencing Consortium, 2001), and approximately 384 000 copies of B1 and 328 000 copies of B2 are present per haploid rat genome (Rat Genome Sequencing Project Consortium, 2004). Retrotransposition is believed to account for SINE amplification and dispersal throughout the genome. Despite the considerable proportion of genetic material assigned to these SINE families, little is known about the function, if any, of their encoded transcripts. It has been proposed that some SINE families are involved in cellular stress responses, as several stress stimuli, including heat shock and DNA-damaging agents, induce the production of SINE transcripts (Li et al, 1999; Liu et al, 1995; Rudin and Thompson, 2001). In fact, recent studies have demonstrated that the 180 base pair (bp) B2 RNA is involved in the repression of class II gene expression in response to heat shock, through a direct

interaction with pol II (Allen et al, 2004; Espinoza et al, 2004). Furthermore, a role for Alu in mediating the response of human cells to heat shock has also been shown (Chu et al, 1998). This challenges a traditionally held concept that SINEs are merely 'junk DNA'.

1.2.6 Viral genes transcribed by pol III

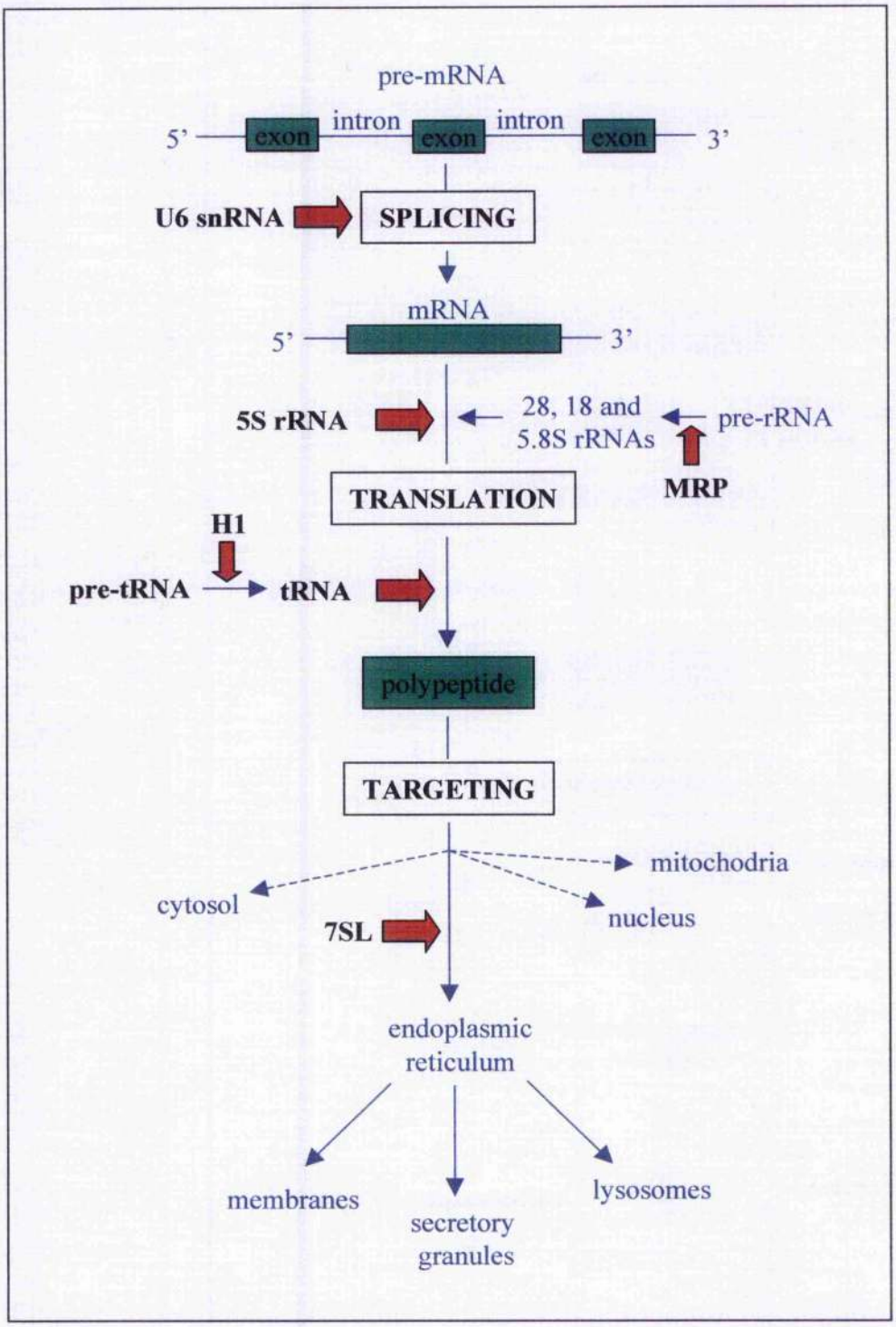
Certain viruses utilise the cellular pol III transcription machinery to transcribe short transcription units within their genomes. For example, the adenovirus VAI and VAII genes are transcribed by pol III (Söderlund et al, 1976; Weinmann et al, 1974). High levels of these transcripts, which are approximately 160 nucleotides in length, are produced during the late stages of adenoviral infection (Akusjärvi et al, 1980; Söderlund et al, 1976; Weinmann et al, 1974). VAI influences host cell protein synthesis to promote the efficient translation of viral mRNAs, thus being essential for the productive expression of the viral genome (Svensson and Akusjärvi, 1984; Thimmappaya et al, 1982). The role of VAII is less clear, as mutants lacking this gene have relatively normal life-cycles (Thimmappaya et al, 1982). However, it has been demonstrated that VAII interacts with and inhibits cellular RNA helicase A, which may be involved in the defence against viral infection (Liao et al, 1998). Similar RNAs are encoded by the Epstein-Barr virus EBER1 and EBER2 genes: these genes share regions of homology with the VA genes, and the resulting RNAs are predicted to have similar secondary structures (Rosa et al, 1981). Furthermore, EBERs can functionally substitute for VAI during adenovirus infection (Bhat and Thimmappaya, 1985). This suggests that EBERs might also act by subverting the host cell translation machinery towards the efficient manufacture of viral

proteins. In addition, transfection of EBERs into mammalian cells has been shown to increase interleukin-10 expression and repress the cellular antiviral interferon response (Kitagawa et al, 2000; Komano et al, 1999). EBERs are sufficient to induce growth in soft agar and tumours in mice, thus providing the first example of an oncogenic RNA (Kitagawa et al, 2000; Komano et al, 1999; Ruf et al, 2000).

Thus, the majority of RNAs synthesised by pol III impinge either directly or indirectly on protein synthesis, and therefore contribute to cell growth capacity. Figure 1.1 illustrates how the best characterised of these class III transcripts participate in various steps leading from mRNA to protein in eukaryotic cells.

The genes discussed above are transcribed solely by pol III. The selective transcription of genes by pols I, II and III is dictated by their distinct promoters, which contain specific sequence elements that direct the recruitment of the appropriate transcription factors and RNA polymerase through multiple protein-DNA and protein-protein interactions. The following section describes the promoter structures specifically recognised by the pol III transcription machinery.

Figure 1.1: Various pol III transcripts are involved in the production and targeting of cellular proteins. Several steps are involved in the route from an initially synthesised pre-mRNA to a functional protein in the appropriate cellular location. Pol III-synthesised RNAs are essential for many of these steps, as indicated in the figure with red arrows. U6 snRNA is required for mRNA splicing as part of the spliceosome, both 5S rRNA and tRNAs directly participate in mRNA translation, and 7SL forms part of the signal recognition particle, which directs nascent polypeptide chains to the endoplasmic reticulum. In addition, the H1 and MRP RNAs are part of ribonucleoprotein particles required for pre-tRNA and pre-rRNA processing, respectively, and thus indirectly contribute to cellular protein synthesis.


pre-mRNA
5'
exon
intron
exon
intron
exon
3'
U6 snRNA
SPLICING
mRNA
5'
3'
5S rRNA
28, 18 and
5.8S rRNAs
pre-rRNA
MRP
TRANSLATION
H1
pre-tRNA
tRNA
polypeptide
TARGETING
mitochodria
cytosol
nucleus
7SL
endoplasmic
reticulum
membranes
secretory
granules
lysosomes

1.3 Class III gene promoters

The promoters utilised by pol III can be divided into three main types. The most notable feature of the majority of these promoters is that the crucial sequence elements are found downstream of the transcription start site, within the transcribed region, rather than upstream, as with class I and II genes. Promoters with such a structure are referred to as type 1 or 2. However, a small minority of pol III-transcribed genes in vertebrates lack any requirement for internal promoter regions, with important sequence elements being found exclusively upstream of the transcription start site. Such promoters are known as type 3. Each of these promoter types is described in more detail below, and depicted in Figure 1.2.

1.3.1 Type 1 promoters

Type 1 promoters are unique to 5S rRNA genes and require three internal promoter elements for efficient transcription: an A-block, an intermediate element (IE) and a C-block (Figure 1.2). The nature of this internal control region (ICR) has been extensively studied in *X. laevis*, revealing that the A-block is located between +50 and +64 relative to the transcription start site, the IE is found at +67 to +72, and the C-block from +80 to +97 (Pieler et al, 1987). Transcriptional efficiency is markedly reduced by mutations in these specific sequence elements or if the spacing between them is altered (Bogenhagen, 1985; Pieler et al, 1985a, b; Pieler et al, 1987).

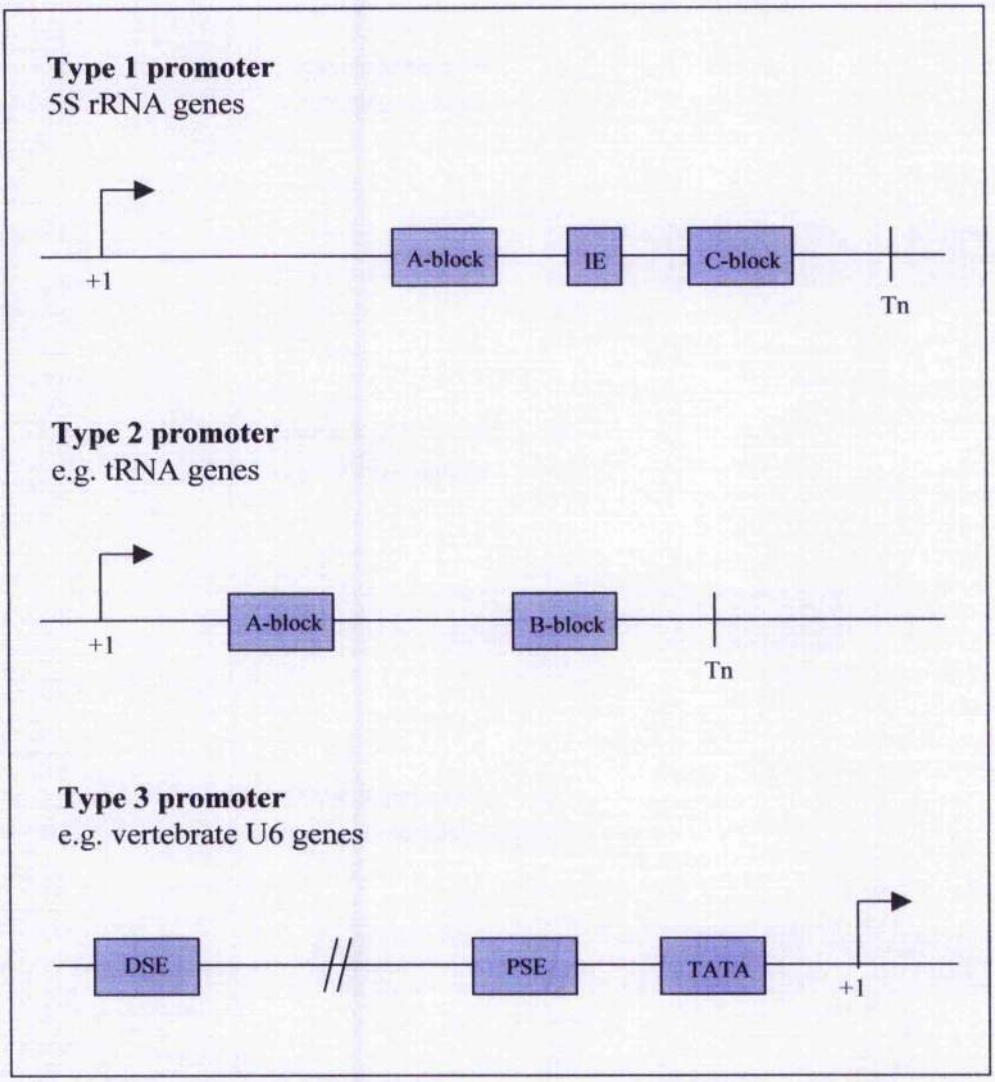


Figure 1.2: Promoter types utilised by pol III. The transcription start site on each promoter is indicated by '+1' and 'Tn' indicates the termination site. The important sequence elements of each promoter type are represented by coloured boxes, and are explained in detail in the text. Abbreviations: DSE, distal sequence element; IE, intermediate element; PSE, proximal sequence element; TATA, TATA box. This figure was adapted from White, 2001.

1.3.2 Type 2 promoters

The majority of pol III transcribed genes, including those for tRNAs, major SINE families and VA RNAs, have type 2 promoters (White, 2002). These consist of two essential, highly conserved sequence elements of about 10bp each: an A-block and a B-block (Figure 1.2; Galli et al, 1981). The A-block is highly homologous to the type 1 promoter A-block, and in some species these elements are functionally interchangeable (Ciliberto et al, 1983). However, in type 2 promoters the A-block is found further upstream, generally within 20bp of the transcription start site (White, 2002). The spacing between the A- and B-blocks is not as restricted as the spacing between type 1 promoter sequence elements. In fact, the position of the B-block is highly variable: an A- to B-block separation of 30-60bp is optimal; however, a distance of up to 365bp can still support transcription (Baker et al, 1987; Fabrizio et al, 1987).

1.3.3 Type 3 promoters

Class III genes with type 3 promoters include the vertebrate U6, 7SK and MRP genes (Das et al, 1988; Murphy et al, 1987; Yuan and Reddy, 1991). In contrast to class III templates with type 1 and 2 promoters, efficient transcription of these genes by pol III requires three extragenic promoter elements: a TATA box, a proximal sequence element (PSE) and a distal sequence element (DSE) (Figure 1.2). The best characterised type 3 promoter is that of a human U6 gene, in which the TATA box, PSE and DSE are found centred approximately 27, 56 and 229bp upstream of the transcription start site, respectively (Das et al, 1988; Kunkel and Pederson, 1988; Kunkel and Pederson, 1989; Lobo and Hernandez, 1989). Although transcribed by different polymerases, the human U6 and U2

(transcribed by pol II) snRNA gene promoters share considerable homology in their DSE and PSE sequence elements (Kunkel and Pederson, 1988). Unlike vertebrates, yeast U6 genes require an ICR with functional A- and B-blocks for transcription, rather than the extragenic elements typical of type 3 promoters. This suggests that type 3 promoters have arisen relatively recently in evolution (Paule and White, 2000).

Thus, each of these distinct promoter types can specify a class III gene and direct its transcription by pol III. However, regardless of the promoter type, pol III itself has little affinity for these promoter sequence elements, and its accurate recruitment to the appropriate genes relies on specific accessory proteins known as transcription factors. The following sections describe the properties and functions of the various components required for the transcription of a class III gene.

1.4 Transcription of class III genes

The process of transcription involves initiation, elongation and termination. Before transcription can proceed, a pre-initiation complex must assemble at the appropriate promoter, ultimately leading to polymerase recruitment.

1.4.1 Transcription initiation complex assembly on class III genes

The route to pol III recruitment varies depending on the promoter type of the gene to be transcribed, as discussed below (and reviewed by Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002).

Type 2 promoters

Figure 1.3 shows a simplified illustration of the formation of a transcription initiation complex on the most commonly used class III gene promoter type (type 2). This process requires two pol III-specific, basal transcription factors: TFIIIC and TFIIIB.

TFIIIC is one of the most complex transcription factors ever studied, having six subunits in yeast, with an aggregate mass of more than 500 kDa (Geiduschek and Kassevetis, 2001; Paule and White, 2000; Schramm and Hernandez, 2001). Each of these subunits has been cloned from *S. cerevisiae* and shown to be essential for cell viability. In isolation, these subunits cannot specifically bind DNA. However, when combined to form TFIIIC, the resulting complex specifically recognises and binds directly to the A- and B-block elements of type 2 promoters. In fact, photocrosslinking experiments have revealed that this enormous and flexible transcription factor can span the entire length of a tRNA

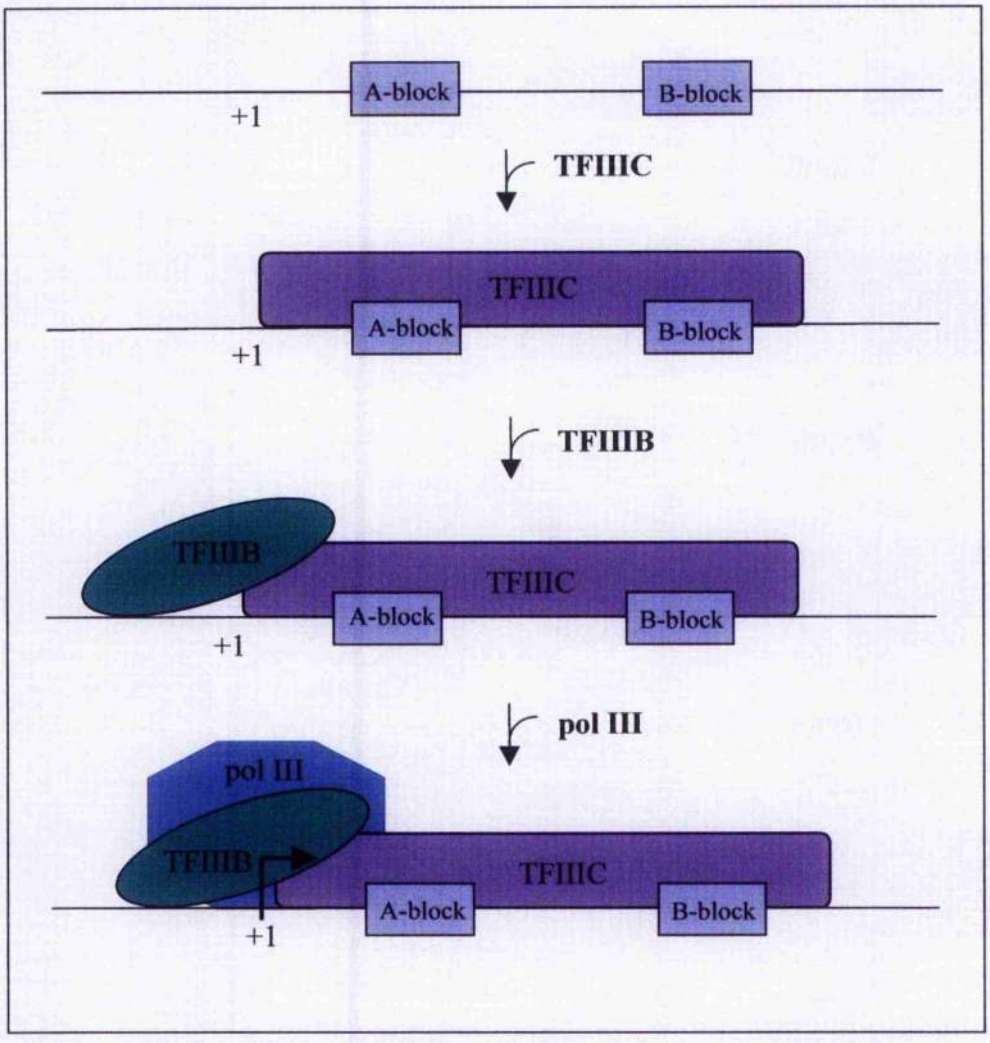


Figure 1.3: Pol III transcription initiation complex assembly on a type 2 promoter. TFIIIC recognises and binds the A- and B-block promoter elements directly. Once bound to the DNA, TFIIIC then recruits TFIIIB via protein-protein interactions. TFIIIB can then recruit pol III to the promoter and transcription is initiated. The transcription start site is indicated by '+1'. This figure was adapted from White, 2002.

gene (Bartholemew et al, 1990). Although both A- and B-blocks are contacted by TFIIIC, the latter is the predominant determinant of binding affinity (Baker et al, 1986).

Human TFIIIC was originally described as an activity in a HeLa cell phosphocellulose fraction required for class III gene expression (Segall et al, 1980), which could be further resolved into two separate components termed TFIIIC1 and TFIIIC2 (Yoshinaga et al, 1987). Both of these are required for transcription from type 1 and 2 promoters *in vitro*; however, only the TFIIIC1 fraction is required by type 3 promoters (Lagna et al, 1994; Oettel et al, 1997; Yoon et al, 1995). The identity of TFIIIC1 is unknown, and a precise function for this factor has yet to be demonstrated unequivocally. In contrast, TFIIIC2 has been well characterised both structurally and functionally, and plays an orthologous role to the yeast TFIIIC factor, in that it binds type 2 promoter DNA directly (Geiduschek and Kassavetis, 2001; Kovelman and Roeder, 1992; Schramm and Hernandez, 2002; Yoshinaga et al, 1989). Despite this, there is little sequence homology between most of the TFIIIC subunits from these species. Human TFIIIC2 is composed of 5 polypeptides, known as TFIIIC220, 110, 102, 90 and 63, according to their molecular masses (Kovelman and Roeder, 1992). TFIIIC220 and 110 primarily generate the DNA-binding surface of TFIIIC, with TFIIIC220 interacting specifically with the B-block (Schramm and Hernandez, 2002; Shen et al, 1996; Yoshinaga et al, 1987, 1989). Specific TFIIIC-DNA interactions are also likely to be contributed by TFIIIC63, via the A-block (Hsieh et al, 1999a). TFIIIC90 is pivotal to the integrity of the TFIIIC complex, as it bridges two distinct subdomains formed by the other four subunits

(Hsieh et al, 1999b). In addition, three of these TFIIIC subunits (220, 110 and 90) have been shown to possess histone acetyltransferase (HAT) activity, which may serve to remodel chromatin in the vicinity of class III genes (Hsieh et al, 1999b; Kundu et al, 1999).

Regardless of the species, the key function of TFIIIC is to recruit TFIIIB, positioning this factor just upstream of the transcription start site. TFIIIB consists of three proteins: TATA box-binding protein (TBP) and two TBP-associated factors, known as Brf1 (TFIIB-related factor 1; so-called because of its N-terminal homology to the pol II-specific transcription factor TFIIB) and Bdp1 (Schramm and Hernandez, 2002). In humans, TBP, Brf1 and Bdp1 have apparent molecular masses of approximately 34, 90 and 160kDa, respectively. TBP is also used by the transcription machineries of pols I and II, and is therefore essential for all nuclear transcription in eukaryotes (Cormack and Struhl, 1992). Conversely, Brf1 and Bdp1 are specifically involved in the transcription of class III genes. Brf1 forms a tight association with TBP in solution; however, Bdp1 is only weakly associated with this complex, if at all, in the absence of a DNA template (Geiduschek and Kassavetis, 2001; Huet et al, 1994; Kassavetis et al, 1991; Schramm and Hernandez, 2002; Schramm et al, 2000). Several alternatively spliced Bdp1 cDNAs have been identified in human cells (Schramm et al, 2000). The involvement of each of these in pol III transcription is unclear. However, one of these splice variants encodes a protein of approximately 250kDa, which has been proposed to constitute at least part of the uncharacterised TFIIIC1 activity mentioned above (Weser et al, 2004).

The recruitment of TFIIIB to class III gene promoters by TFIIIC has been extensively studied in *S. cerevisiae*. During transcription initiation complex formation, DNA-bound TFIIIC initially contacts the Brf1 subunit of TFIIIB, and this is thought to occur via the *S. cerevisiae* equivalent of human TFIIIC102 (Kassavetis et al, 1992; Schramm and Hernandez, 2002). Several subsequent interactions that occur between each of the TFIIIB subunits and various TFIIIC components are also likely to participate in the formation of a stable pre-initiation complex (Schramm and Hernandez, 2002). In human cells, it has been confirmed that TFIIIC90 interacts with Brf1, and that TFIIIC102 and 63 interact with both Brf1 and TBP (Hsieh et al, 1999a, b).

TFIIIB is essential for the recruitment of pol III to the transcription start site of class III genes. All three TFIIIB subunits are required for polymerase recruitment; however, only Brf1 and TBP have been shown to make direct contacts (Schramm and Hernandez., 2002). Of these contacts, an interaction between Brf1 and the yeast pol III-specific subunit C34 (RPC39 in humans) appears particularly important (Brun et al, 1997). Furthermore, it has recently been demonstrated that various TFIIIC subunits are capable of interacting with pol III, suggesting that this DNA-binding complex may also contribute to pol III recruitment (Flores et al, 1999; Hsieh et al, 1999a, b).

Type 1 (5S rRNA) promoters

Figure 1.4 shows a transcription initiation complex on a typical 5S rRNA promoter. As with type 2 promoters, the recruitment of pol III relies on TFIIIB, which is positioned just upstream of the transcription start site by TFIIIC.

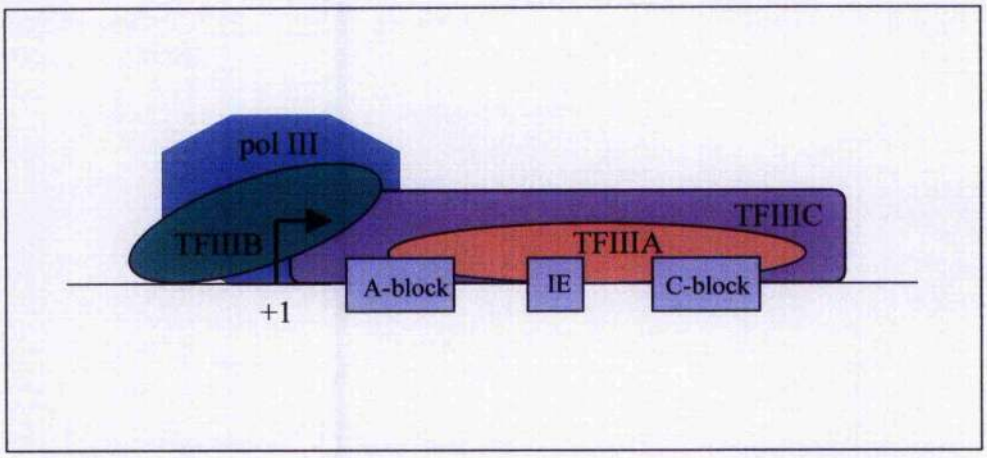


Figure 1.4: Type 1 promoter transcription initiation complex. Recruitment of TFIIIC, and hence TFIIIB and pol III, to type 1 promoters requires TFIIIA, which binds promoter elements within the ICR of 5S rRNA genes as shown. The transcription start site is indicated by '+1'.

However, type 1 promoters lack a functional B-block (Figure 1.2), which is the major determinant of TFIIIC DNA-binding affinity. Consequently, the gene-specific transcription factor TFIIIA is required for the productive association of TFIIIC with 5S rRNA genes (Engelke et al, 1980; Segall et al, 1980). TFIIIA was the first eukaryotic transcription factor to be purified (Engelke et al, 1980) and cloned (Ginsberg et al, 1984). It consists of a single polypeptide of approximately 40kDa, and has nine zinc finger domains that bind the ICR DNA of 5S genes. Three of these zinc fingers specifically recognise the C-block, and this constitutes the majority of TFIIIA DNA-binding energy (Paule and White, 2000). TFIIIA-bound 5S genes are competent to recruit TFIIIC. However, it is unclear how TFIIIA promotes this TFIIIC binding (Schramm and Hernandez, 2002).

Type 3 promoters

As shown in Figure 1.5, TFIIIB also forms part of the transcription initiation complex on vertebrate type 3 promoters. However, the TFIIIB entity involved in transcription from these promoters does not contain Brf1, but instead Bdp1, TBP and a Brf1-related factor known as Brf2 constitute an alternative, type 3 promoter-specific TFIIIB activity (Schramm et al, 2000). Furthermore, the way in which TFIIIB is recruited to the transcription start site differs from type 1 and 2 promoters, and does not require TFIIIC2 or TFIIIA (Lagna et al, 1994; Schramm and Hernandez, 2002). Type 3 promoters contain two upstream sequence elements in the vicinity of the transcription start site: a TATA box and a PSE. These elements are directly recognised by the TBP subunit of TFIIIB, and a five-subunit factor known as $SNAP_c$, respectively (Lobo et al, 1991; Murphy et

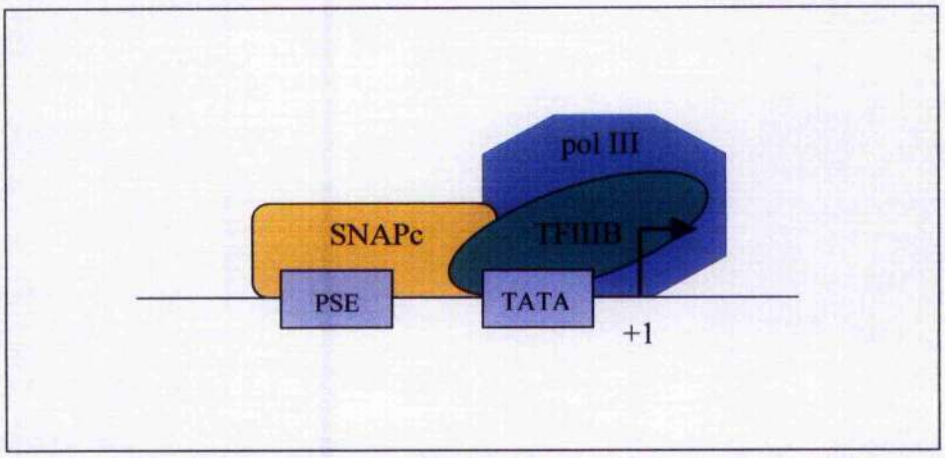


Figure 1.5: Type 3 promoter transcription initiation complex in the vicinity of the transcription start site. TFIIIB and $SNAP_c$ bind cooperatively to the TATA box and PSE of type 3 promoters, respectively. Interactions between DSE-bound Oct-1 and $SNAP_c$ (not shown) enhance this $SNAP_c$/TFIIIB recruitment. Pol III is recruited to the transcription start site (indicated by '+1') following $SNAP_c$/TFIIIB binding, and transcription commences.

al, 1992). Alone, TFIIIB and $SNAP_c$ bind poorly to DNA; however, protein-protein interactions between these complexes enhance their recruitment to the promoter (Mittal and Hernandez, 1997). In addition, the DSE of type 3 promoters (found further upstream, see Figure 1.2) binds an additional transcription factor known as Oct-1, which further stimulates TFIIIB/$SNAP_c$ recruitment through protein-protein interactions between Oct-1 and $SNAP_c$ (Mittal et al, 1996; Murphy et al, 1992). However, although stimulatory, Oct-1 is not essential for basal transcription from type 3 promoters *in vitro* (Hu et al, 2003). Once TFIIIB/$SNAP_c$ have assembled on the promoter, pol III can be recruited. The interactions responsible for pol III recruitment to these promoters have yet to be defined.

Therefore, the components of the pre-initiation complexes utilised by pol III vary depending on the promoter type. However, TFIIIB is essential for polymerase recruitment to, and hence the transcription of, all class III genes. Therefore, TFIIIB is regarded as the central pol III transcription initiation factor.

1.4.2 Pol III

Pol III is a huge 600-700kDa complex composed of 17 subunits in yeast and humans, making it the largest of the three eukaryotic nuclear RNA polymerases (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002). Genetic studies in yeast have indicated that at least 16 of these 17 subunits are essential for pol III function and yeast viability (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002). Five of the 17 subunits are

shared by all three RNA polymerases, a further two are common to both pols I and III, and ten subunits are unique to the pol III complex (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002). All three polymerases perform the same fundamental function: they catalyse the covalent attachment of ribonucleotides to form an RNA chain that is complementary to the gene template being transcribed. Therefore, it is not surprising that these enzymes share some common subunits. However, the polymerases transcribe distinct genes with different characteristics, and so they also have several unique properties. The subunits unique to pol III are presumed to contribute to pol III-specific properties, such as its distinct nucleoplasmic localisation (Pombo et al, 1999), its interaction with specific transcription initiation factors (as described above), and its ability to perform elongation and termination of transcription independently of accessory factors (as discussed below).

1.4.3 Transcription initiation, elongation and termination by pol III

Following pol III recruitment, the two strands of DNA around the transcription start site are separated to form a transcription bubble (Geiduschek and Kassavetis, 2001; White, 2002). This melting of the DNA helix allows the polymerase to access the template strand, and is required before transcription can proceed. DNA melting is performed by the polymerase, although the Brf1 and Bdp1 components of TFIIIB also play an active role (Geiduschek and Kassavetis, 2001; Kassavetis et al, 1998, 2001; Schramm and Hernandez, 2002). Thus, TFIIIB serves not only to recruit pol III, but also participates in the formation of an open promoter complex.

Once the DNA strands have been separated, RNA synthesis can be initiated, and the polymerase progresses into the gene and dissociates from promoter-bound TFIIIB (White, 2002). The transcription bubble moves downstream with the elongating polymerase. Although TFIIIC assembles within the transcribed regions of the majority of class III genes, this large factor is not dissociated from promoters during elongation (White, 2002). It is unclear at present how the polymerase passes DNA-bound TFIIIC during transcription. However, unlike pols I and II, pol III does not require any accessory factors for efficient chain elongation (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002). Furthermore, termination by pol III also occurs independently of other factors: four or more T residues within the template strand of a class III gene are sufficient to signal the accurate and efficient termination of transcription (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002).

Following the synthesis of the first transcript, pol III is known to be recycled on the same DNA template for several further rounds of transcription (Dieci and Sentenac, 1996). As a consequence, the slow initial step of polymerase recruitment is avoided, making the production of subsequent RNAs by pol III more efficient (Dieci and Sentenac, 1996). TFIIIB participates in this pol III recycling and under certain circumstances, TFIIIC is also required (Ferrari et al, 2004).

1.5 Pol III transcription can be stringently regulated

Many pol III products are involved in cellular biosynthesis, as detailed in section 1.2, and are therefore constitutively available to perform a variety of 'housekeeping' functions. However, the levels of these transcripts vary considerably in accordance with changing metabolic requirements as a result of tightly regulated pol III transcription. For example, pol III transcription is low when nutrients or mitogens are limiting, but upregulated when the availability of growth stimuli increase (Clarke et al, 1996; Johnson et al, 1974; Mauck and Green, 1974; Sethy et al, 1995). Furthermore, growth inhibitory agents, such as cyclohexamide and rapamycin, downregulate the production of tRNA and 5S rRNA, whereas growth-promoting agents, such as phorbol esters, induce pol III transcription (Garber et al, 1991; Gokal et al 1986; Zaragoza et al, 1998). Pol III transcription is also regulated in response to other cellular stresses, throughout the cell cycle, and during metazoan cellular differentiation and development (as outlined in the following sections). Various mechanisms contributing to this stringent control have been described, some of which are discussed below.

1.5.1 Regulation of pol III transcription in proliferating cells

The regulation of pol III transcription has been particularly well characterised in proliferating mammalian cells. Mitogenic stimulation of quiescent eukaryotic cells results in the activation of several intracellular signalling cascades, which can initiate a proliferative response. Some of these cascades have been shown to mediate an accompanying activation of pol III transcription. For example, CK2, a highly conserved growth-promoting eukaryotic protein kinase, has been shown to phosphorylate and activate TFIIIB, thus increasing pol III transcription, in both

yeast and mammalian cells (Ghavidel and Schultz, 1997; Hockman and Schultz, 1996; Johnston et al, 2002). Furthermore, the mitogen-activated extracellular signal-regulated kinase (ERK), which is activated via the ras signalling pathway in mammalian cells, can induce pol III transcription through phosphorylation of the Brf1 component of TFIIIB (Felton-Edkins et al, 2003a). In addition to these kinases, the proto-oncogene product c-Myc is a crucial regulator of cell growth and proliferation in metazoans (Grandori et al, 2000; Schmidt, 1999). c-Myc specifically activates pol III transcription, again by targeting TFIIIB, leading to an increase in tRNA and 5S rRNA production (Felton-Edkins et al, 2003b; Gomez-Roman et al, 2003).

Another important regulator of mammalian proliferation is the retinoblastoma protein (RB). One of the main functions of RB is to ensure that cells only progress through the cell cycle and divide in response to the appropriate external stimuli (Kaelin, 1999; Weinberg, 1995). Loss of RB function results in aberrant cellular proliferation and tumour formation (Sherr, 1996). RB is therefore known as a tumour suppressor protein. In resting cells, RB is primarily found in an underphosphorylated form capable of interacting with several cellular targets, including TFIIIB (Scott et al, 2001). RB-bound TFIIIB is unable to interact with other components of the pol III transcription machinery, and thus in this way, RB represses class III gene expression (Sutcliffe et al, 2000). However, in response to growth stimuli, the cell cycle regulatory proteins cyclins D and E accumulate and activate their specific cyclin-dependent kinase (CDK) partners, which in turn phosphorylate and inactivate RB (Hulleman and Boonstra, 2001). This is essential for progression into the DNA synthesis (S) phase of the cell cycle.

Hyperphosphorylated RB dissociates from TFIIIB leading to the activation of pol III transcription and the accumulation of class III gene products prior to S phase entry (Scott et al, 2001). RB also regulates the transcription of certain genes by pol II, whose products are involved in DNA synthesis (Dyson, 1998), and transcription by pol I, which synthesises the large rRNAs (Cavanaugh et al, 1995). Therefore, this common regulatory mechanism allows components of the DNA and protein synthetic machinery to be produced in a highly coordinated manner during the cell cycle. Following S phase, pol III transcription declines as cells reach mitosis. Again, TFIIIB appears to be the primary target for regulation; in this case, TFIIIB activity is inhibited by direct phosphorylation (Fairley et al, 2003; Gottesfeld et al, 1994; Hu et al, 2004; Leresche et al, 1996; White et al, 1995a).

1.5.2 Regulation of pol III transcription in response to cellular stresses

Various cellular insults, including genotoxic stress and nutrient deprivation, repress pol III transcription (Clarke et al, 1996; Crighton et al, 2003; Ghavidel and Schultz, 2001; Sethy et al, 1995; Upadhya et al, 2002). In mammalian cells, this is thought to be partly mediated by p53, which can induce cell cycle arrest or cell death in response to a variety of stresses including radiation, hypoxia and oncogenic stimuli (Vousden, 2000). As with RB, p53 is a tumour suppressor protein whose function is compromised in most human tumours (Vousden, 2000). Although unrelated to RB, p53 also represses pol III transcription by binding and inactivating TFIIIB (Cairns and White, 1998).

Another negative effector of pol III transcription is Maf1. This protein was recently identified in *S. cerevisiae*, and shown to interact with TFIIIB and pol III (Pluta et al, 2001; Upadhya et al, 2002). Maf1 represses pol III transcription in response to a range of cellular stresses, such as nutrient limitation and DNA damage (Upadhya et al, 2002). Although homologues of Maf1 have been identified in higher eukaryotes (Pluta et al, 2001), a role for Maf1 in the regulation of pol III transcription has thus far only been demonstrated in yeast.

As mentioned above, CK2 is known to activate pol III transcription under normal growth conditions (Ghavidel and Schultz, 1997; Hockman and Schultz, 1996; Johnston et al, 2002). It was recently demonstrated that CK2 is also involved in transducing DNA damage signals to the pol III transcription machinery in yeast (Ghavidel and Schultz, 2001). Stimulatory CK2 activity is a complex of two catalytic and two regulatory subunits. In response to genotoxic stress, the catalytic subunits are released. The residual regulatory subunits remain bound to TFIIIB, but can no longer phosphorylate and activate this factor, thus resulting in the repression of class III gene expression (Ghavidel and Schultz, 2001; Schultz, 2003). The involvement of CK2 in the downregulation of pol III transcription under DNA-damaging conditions in mammalian cells has not yet been described.

1.5.3 Regulation of pol III transcription during differentiation

Cellular differentiation is accompanied by specific changes in gene expression, cellular morphology and a reduction or cessation of proliferation. Cell culture systems, which mimic the processes accompanying differentiation, have been used to investigate this developmental phenomenon. For example, F9 embryonal

carcinoma cells can be induced to differentiate into cells resembling parietal or visceral endoderm, depending on the conditions used (Hogan et al, 1981; Strickland et al, 1980). Such differentiation is accompanied by a specific decrease in the level of class III transcripts (Meißner et al, 1995; Murphy et al, 1983; White et al, 1989). In the case of parietal endoderm formation, this was shown to result from a decrease in TFIIIB activity, which could be at least partly attributed to a decline in the level of Brf1 (Alzuherri and White, 1998; White et al, 1989). Tight developmental regulation of pol III transcription has also been suggested by *in situ* hybridisation analysis of class III transcripts at various stages of early mouse embryogenesis (Vasseur et al, 1985). However, the regulation of pol III transcription in response to growth stimulation of differentiated cells has not been investigated.

1.5.4 Aberrant activation of pol III transcription in transformed cells

The precise regulation of pol III transcription is clearly a prominent feature of normal cellular growth and proliferation. In contrast, deregulated pol III transcription accompanies the aberrant proliferation characteristic of many transformed and tumour cell types (reviewed by White, 2004a, b). Various mechanisms have been proposed to cause this subversion of normal pol III transcriptional control. For example, as discussed above, pol III transcription can be restrained by two key tumour suppressors, p53 and RB, and activated by known proto-oncogene products, including CK2, ras and c-Myc. Inactivation of p53 and RB, and abnormal activation of proto-oncogenes, are common features of most human cancers, and are likely to contribute to the elevation of pol III transcripts frequently observed in tumour cells (White, 2004a, b). Furthermore,

pol III transcription is stimulated by several viruses associated with cellular transformation and human cancers, through the activation of TFIIIB and/or TFIIIC (White, 2004a, b). Abnormally elevated expression of TFIIIB and TFIIIC subunits have been described in certain virally-transformed cells and tumour types, and this may contribute to the deregulation of class III gene expression under these circumstances (Daly et al, 2004, *In press*; Felton-Edkins and White, 2002; Larminie et al, 1999; Wang et al, 1995; Winter et al, 2000). Therefore, various aberrations in cellular control mechanisms ensure pol III transcription is elevated in tumours, suggesting that such elevation is advantageous for rapid growth and proliferation.

The existence of a complex network of pol III regulatory mechanisms, as outlined above, suggests that the stringent control of class III gene expression is an important aspect of proper cellular function, and is likely to be fundamental to the appropriate regulation of cellular biosynthetic capacity and ultimately cell growth.

1.6 Aims of PhD

The overall objective of this PhD was to investigate the regulation of pol III transcription, specifically during cardiomyocyte hypertrophy. As mentioned previously, the hypertrophic growth of cardiomyocytes occurs in the absence of cell division and is associated with cardiovascular disease, which is highly prevalent in many countries. Therefore, research into the mechanisms underlying this response is of considerable relevance to the fundamental appreciation of cell growth controls and numerous cardiac pathologies. The current understanding of cardiomyocyte hypertrophy is outlined in Chapter 3. One of the key hallmarks is an increase in protein synthesis, which underlies cell growth; however, the mechanisms responsible for this increased translation are not fully understood. As discussed in this introduction, pol III manufactures essential components of the cellular protein synthetic machinery (see Figure 1.1), and the production of these RNAs is subject to stringent control under a variety of cellular conditions. Therefore, it seemed likely that pol III transcriptional regulation would be a critical feature of the hypertrophic growth response. The first aim of the project was to determine whether cardiomyocyte hypertrophy is accompanied by enhanced pol III transcription. To date, investigations on pol III transcriptional activation in mammals have focused solely on undifferentiated, proliferating cell types. Thus, the remainder of the investigation was concerned with elucidating the mechanisms responsible for upregulating pol III transcription in these terminally differentiated cells, with a view to characterising pathways directly involved in inducing a disease-associated cell growth response, through increased class III gene expression.

CHAPTER 2
Materials and Methods

2.1 Cell culture

2.1.1 Cardiomyocytes

Primary cultures of rat neonatal cardiomyocytes were prepared as follows. Hearts were excised from 1- to 5-day-old Sprague Dawley rats by Central Biological Services (Institute of Biomedical and Life Sciences, University of Glasgow), and supplied in ADS buffer (120 mM NaCl, 20 mM HEPES, 0.8 mM NaH_2PO_4, 5 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, 100 units/ml penicillin and 100 µg/ml streptomycin, pH 7.35). All subsequent procedures were performed using aseptic technique and sterile equipment and reagents. Hearts were transferred to a petri dish on ice containing chilled ADS buffer in a class II hood. Ventricular tissue was isolated from the hearts using forceps, then minced using a razor blade. Minced tissue was transferred to a falcon tube, and excess ADS buffer was removed. Myocytes were dissociated from ventricular tissue by multiple rounds of enzymatic digestion at 37°C, with gentle shaking, using 0.48 mg/ml collagenase (Worthington) and 0.6 mg/ml pancreatin (Sigma) dissolved in ADS buffer. Depending on the amount of starting material, 4 to 6 x 15 minute digestions were required to completely dissociate the tissue into its constituent cells. Chilled foetal calf serum (FCS) was used to inactivate the enzymes at the end of each digestion. Dissociated cells were separated from the enzyme/FCS solution by centrifugation at 350g (in a benchtop Labofuge 400R, Heraeus Instruments) for 10 minutes at 4°C. The enzyme solution was removed and the

cells were washed once in Dulbecco's modified Eagle's medium/medium 199 (DMEM/M199 at a ratio of 4:1 v/v) supplemented with 10% horse serum (HS), 5% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin (all Sigma), again by centrifugation at 350g for 10 minutes. The cells were then resuspended in the same medium and pre-plated for 1 hour at 37°C in a humidified, 5% CO_2 incubator, on uncoated 100mm tissue culture dishes. This step was performed to separate myocytes, which do not adhere to uncoated dishes, from non-myocytes, which do. After 1 hour, unattached myocytes were removed and plated on to 24-well, 6-well or 100mm tissue culture dishes, that had been coated with a 1% (w/v) gelatin (Sigma) solution, in the medium described above. Cells were plated at a density of approximately 10^3 cells per mm^2. After 24 hours, confluent myocytes were beating spontaneously. At this time, cells were washed in DMEM/M199 (4:1) supplemented with 4% HS, 100 units/ml penicillin and 100 μg/ml streptomycin (maintenance medium), then maintained in this medium until use (usually 24 hours).

For experiments, cardiomyocytes were serum-starved in DMEM/M199 (4:1) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. FCS was used at a final concentration of 10%. Endothelin-1 (ET-1) and phenylephrine (PE) (both Sigma) were dissolved in sterile distilled H_2O. ET-1 was used at a final concentration of 100 nM, and PE was used at a final concentration of 100 μM. PD98059 (Calbiochem), 5-bromodeoxyuridine (BrdU) (Sigma) and MG132 (Calbiochem) were dissolved in dimethylsulphoxide (DMSO) and used at final concentrations of 50 μM, 0.1 mM and 10 μM, respectively. Where DMSO was

used as a vehicle, final DMSO concentrations were 0.1% in the tissue culture medium.

2.1.2 Rat 1A fibroblasts

As above, rat 1A cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents. Rat 1A fibroblasts were maintained in DMEM supplemented with 5% FCS, 400 µg/ml Geneticin, 100 units/ml penicillin and 100 µg/ml streptomycin (all Sigma), and incubated in serum-free medium for 24 hours prior to stimulation with 10% FCS. Cells were passaged when subconfluent (approximately every 2 to 3 days) using buffered trypsin (0.05% trypsin, 0.02% EDTA; Sigma). Cells were also stored by cryo-freezing. For cryo-freezing, trypsinised cells were resuspended in 70% DMEM (plus penicillin and streptomycin), 20% FCS and 10% DMSO. 1 ml aliquots were transferred to cryo-tubes, and frozen overnight at -80°C, before transferring to liquid nitrogen for permanent storage.

2.2 RNA extraction

2.2.1 RNA extraction from cultured cells

Total cellular RNA was extracted from cardiomyocytes grown on 6-well tissue culture dishes using TRI reagent (Sigma), according to the manufacturer's instructions. Media was aspirated from the cells, then cells were scraped into TRI reagent and transferred to sterile 1.5 ml microfuge tubes. 1 ml of TRI reagent was used per 2 to 3 wells of a 6-well plate. Samples were incubated for 5 minutes at room temperature, to allow the complete dissociation of nucleoprotein

complexes, then 0.2 ml of chloroform was added to each. Thorough mixing of chloroform and TRI reagent was ensured by vortexing each sample for 15 seconds. Samples were incubated at room temperature for a further 5 minutes, then centrifuged (in an Eppendorf Centrifuge 5415R) at 16000g for 15 minutes at 4°C. This centrifugation separated the samples into 3 phases: a lower organic phase containing proteins, a middle interphase containing DNA, and an upper aqueous phase containing RNA. The RNA-containing phase was carefully removed, and transferred to a fresh microfuge tube. To precipitate RNA, 0.5 ml of isopropanol was added, samples were mixed by vortexing for 10 seconds, then incubated for 10 minutes at room temperature. Subsequently, samples were centrifuged at 16000g for 10 minutes at 4°C. Following centrifugation, the supernatant was discarded, and the remaining RNA pellet was washed using 1ml of 75% ethanol, made using diethylpyrocarbonate (DEPC)-treated H_2O (0.1% DEPC). Samples were centrifuged for a further 5 minutes at 16000g (4°C), then the supernatant was aspirated off, and RNA pellets left to air dry for approximately 10 minutes. Once dry, RNA was resuspended in 10-30 µl of DEPC-treated H_2O. To aid resuspension, pre-warmed DEPC-treated H_2O was used, and samples were incubated at 65°C for 15 minutes. Following resuspension, a spectrophotometer was employed to measure the absorbance of each sample at 260 nm, and the following formula was used to calculate the RNA concentration: RNA concentration (µg/ml) = absorbance at 260 nm x 40 x dilution factor. All RNA samples were stored at -80°C.

2.2.2 RNA extraction from whole hearts

Total RNA was also extracted from tissue samples, all of which were obtained from Dr WR MacLellan, Cardiovascular Research Laboratories, Department of Physiology, University of California at Los Angeles School of Medicine, USA. Animals were handled and tissue removed according to institutional guidelines. Following removal, hearts were immediately snap-frozen in liquid nitrogen, and shipped on dry ice. Tissue was crushed using a pestle and mortar in liquid nitrogen, then transferred to sterile microfuge tubes. 1 ml of TRI reagent was added per 50-100 mg of tissue, then tissue homogenised using a Polytron homogeniser. Subsequently, the homogenate was centrifuged at 12000g for 5 minutes to remove any insoluble material. The supernatant (containing RNA) was transferred to a fresh microfuge tube, and RNA was extracted as outlined in section 2.2.1 above.

2.3 Northern blot analysis

10 μg of RNA was diluted in DEPC-treated H_2O to give a total volume of 10 μl. A further 10 μl of 2 X RNA sample buffer (1 X MOPS [20 mM MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA (pH 8.0)], 4.4 M formaldehyde, 54% formamide) was added to this, then samples were incubated at 65°C for 15 minutes to denature RNA secondary structure. Subsequently, samples were immediately cooled on ice to prevent any renaturation of the RNA. 2 μl of 1 mg/ml ethidium bromide and 2 μl of 10 X RNA loading dye (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) were added to each sample, prior to loading onto a denaturing formaldehyde 1% agarose gel (1%

agarose, 2.2M formaldehyde, 1 X MOPS) that had been pre-run at 40V in 1 X MOPS for 30 minutes. Electrophoresis was performed for 4 to 5 hours at 40V in 1 X MOPS, then the gel was visualised under a UV transilluminator to ensure effective RNA separation and equal loading.

The gel was then washed in 20 X SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0) with gentle shaking for 10 to 15 minutes. For capillary transfer of the RNA, the gel was inverted and placed on a wick of Whatmann 3MM chromatography paper, which had been pre-soaked in and was fed from a resevoir of 20 X SSC. A gel-sized piece of pre-soaked Hybond N nylon membrane (Amersham), followed by 2 pieces of pre-soaked Whatmann paper, were placed on the gel ensuring no air bubbles between layers. A stack of folded paper towels was then added followed by a 0.5 kg weight, to ensure efficient transfer of RNA to the membrane by capillary action. Pieces of autoradiography film were placed at the edges of the gel to prevent the paper towels from contacting the wick. The transfer was allowed to proceed for 16 to 18 hours, and then RNA was fixed to the membrane by UV crosslinking at 1200 μJ. The membrane was rinsed for 5 minutes in DEPC-treated H_2O and stored in Saran wrap until use.

To specifically detect the RNA of interest, radiolabelled complementary DNA probes were used: the B2 gene probe was prepared from a 240bp EcoRI-PstI fragment of pTB14, and the probe for acidic ribosomal phosphoprotein P0 (ARPP P0) was prepared from a 1kb EcoRI-HindIII fragment of the mouse cDNA. A Megaprime DNA Labelling Kit (Amersham) was used to label the probes by random oligonucleotide priming, according to the manufacturer's

instructions. Random hexamer oligonucleotides were mixed with 25 ng of the DNA fragment to be probed, and made up to a total volume of 50 µl with DEPC-treated H_2O. This was heated at 95°C for 5 minutes to denature the DNA. Slow cooling of the mixture to room temperature allowed the random hexamer oligonucleotides to anneal to the DNA. 10 µl of reaction buffer (dATP, dGTP, dTTP in Tris pH 7.5, β-mercaptoethanol and $MgCl_2$), 2 µl (2U) of DNA polymerase I Klenow fragment, and 50 µCi of [α-^{32}P] dCTP (Amersham) were added, and labelling was allowed to proceed at 37°C for 1 hour. The labelled DNA was then denatured by heating at 100°C for 5 minutes, and then stored on ice until use.

Prior to hybridising the membrane with an appropriate radiolabelled probe, it was pre-hybridised in a hybridisation oven at 45°C for 45 minutes by rotating in 25 ml of hybridisation buffer (0.2 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 1% bovine serum albumin (BSA), 7% sodium dodecyl sulphate (SDS), 45% formamide in DEPC-treated H_2O). The radiolabelled probe was then added to 25 ml of fresh hybridisation buffer, and incubated with the membrane overnight at 45°C with rotation. The following day, the membrane was washed with rotation in hybridisation wash buffer (40 mM sodium phosphate buffer pH 7.2, 1 mM EDTA and 1% SDS) twice for 5 minutes at 65°C, then twice for 15 minutes at 65°C, to remove any non-specific radioactivity. The membrane was then exposed to autoradiography film for an appropriate length of time at –80°C. To reprobe the membrane, it was boiled in DEPC-treated H_2O for 4 minutes, then pre-hybridised and probed as before.

2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)

2.4.1 Preparation of cDNAs

3 μg of RNA (or 1.2 μg if derived from tissue, see below) and 200 ng of random hexanucleotide primers (Roche) were diluted in DEPC-treated H_2O to give a final volume of 24 μl. Primer annealing was carried out for 10 minutes at 80°C. Following this, samples were transferred to ice then 1 μl (200 U) of Superscript II reverse transcriptase (Invitrogen Life Technologies), 8 μl of 5 X First Stand Buffer (Invitrogen Life Technologies), 4 μl of 0.1 M dithiolthreitol (DTT) (Invitrogen Life Technologies) and 2 μl of a mix containing 10 mM of each of the four dNTPs (Promega) were added to each. Reverse transcription was then allowed to proceed for 1 hour at 42°C. The reaction was stopped by heating at 70°C for 15 minutes. cDNAs were stored at –20°C.

RNA derived from tissue was DNase I-treated prior to cDNA synthesis using a DNA-*free* kit (Ambion), according to the manufacturer's instructions. 10 μg of RNA was diluted with DEPC-treated H_2O to give a final volume of 25 μl. To this, 2.5 μl of 10 X DNase I Buffer (Ambion) and 1 μl (2 U) of DNase I (Ambion) were added. Samples were incubated at 37°C for 30 minutes. To inactivate the DNase I, 5 μl of DNase Inactivation Reagent slurry (Ambion) was added, and samples incubated for 2 minutes at room temperature. Subsequently, samples were centrifuged (in an Eppendorf Centrifuge 5415R) at 10000g for 1 minute to pellet the Inactivation Reagent. The supernantant containing DNA-free RNA was transferred to a fresh sterile microfuge tube, and stored at –80°C. 1.2 μg of this RNA was used for cDNA synthesis, as described above.

2.4.2 PCR

PCRs were performed using a Techgene thermal controller (TECHNE). Each reaction had a total volume of 20 µl and contained 2 µl of cDNA, 20 pmol of the appropriate primers, 0.5 U of *Taq* DNA polymerase (Promega), 1 X *Taq* DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each non-radioactive dNTP, and 1.8 µCi of [α-^{32}P] dCTP (Amersham). Table 2.1 lists the primers used for PCR, and their sequences. The cycling parameters employed and product size obtained for each primer set are indicated in Table 2.2. Reaction products were diluted 1:1 with formamide loading buffer (98% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 5 mM EDTA), and resolved on 7% polyacrylamide sequencing gels containing 7 M urea and 0.5 X TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run at 40W for 30 minutes in 0.5 X TBE prior to loading samples (1.5 µl of each). Before loading, samples were heated at 95°C for 2 minutes, then quenched on ice. Electrophoresis was carried out for 1 hour at 40W in 0.5 X TBE, then gels were vacuum-dried for 1 hour at 80°C. Radiolabelled PCR products were visualised by autoradiography.

Table 2.1: PCR primers

Transcript	Forward (F) and reverse (R) primers
ARPP P0	F: 5'-GCA CTG GAA GTC CAA CTA CTT C-3' R: 5'-TGA GGT CCT CCT TGG TGA ACA C-3'
$tRNA^{Leu}$	F: 5'-GTC AGG ATG GCC GAG TGG TGT AAG GCG CC-3' R: 5'-CCA CGC CTC CAT ACG GAG ACC AGA CCC-3'
$tRNA^{Tyr}$	F: 5'-CCT TCG ATA GCT CAG CTG GTA GAG CGG AGG-3' R: 5'-CGG AAT TGA ACC AGC GAC CTA AGG ATG TCC-3'
$tRNA^{Arg}$	F: 5'-GGC TCT GTG GCG CAA TGG ATA-3' R: 5'-TTC GAA CCC ACA ACC TTT GAA TTG CTC-3'
5S rRNA	F: 5'-GGC CAT ACC ACC CTG AAC GC-3' R: 5'-CAG CAC CCG GTA TTC CCA GG-3'
U6 snRNA	F: 5'-GCT CGC TTC GGC AGC ACA TAT AC-3' R: 5'-TAT CGA ACG CTT CAC GAA TTT GCG-3'
Brf1	F: 5'-CTA CTT GGT TTG CCG AAC G-3' R: 5'-TCC TCT GTC CAG CGG TGT A-3'
atrial natriuretic factor (ANF)	F: 5'-ATG GGC TCC TTC TCC ATC AC-3' R: 5'-TCT TCG GTA CCG GAA GCT G-5'
c-Myc	F: 5'-CCA GTG AGG ATA TCT GGA AG-3' R: 5'-TTC ACC ATG TCT CCT CCA AG-3'
cyclin D2	F: 5'-TTA CCT GGA CCG TTT CTT GG-3' R: 5'-TGC TCA ATG AAG TCG TGA GG-3'
nucleolin	F: 5'-CGC GTC CGA GGC AGT G-3' R: 5'-TCC ATC TAC CGT CAC GGT CAG-3'

Table 2.2: PCR cycling parameters and product sizes

Transcript	Cycling parameters	Product size
ARPP P0	95°C for 2 minutes, 18 to 22 cycles of [95°C for 1 minute, 58°C for 30 seconds, 72°C for 1 minute], 72°C for 3 minutes.	265bp
$tRNA^{Leu}$	95°C for 3 minutes, 25 to 29 cycles of [95°C for 30 seconds, 68°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.	88bp
$tRNA^{Tyr}$	95°C for 3 minutes, 25 to 29 cycles of [95°C for 1 minute, 62°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.	84bp
$tRNA^{Arg}$	95°C for 2 minutes, 30 cycles of [95°C for 30 seconds, 65°C for 30 seconds, 72°C for 15 seconds], 72°C for 5 minutes.	74bp
5S rRNA	95°C for 3 minutes, 18 to 22 cycles of [95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.	107bp
U6 snRNA	95°C for 3 minutes, 25 cycles of [95°C for 1 minute, 60°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.	96bp
Brf1	95°C for 5 minutes, 21 to 25 cycles of [95°C for 1 minute, 61°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.	490bp
ANF	94°C for 1 minute, 20 to 25 cycles of [94°C for 45 seconds, 63°C for 45 seconds, 72°C for 90 seconds], 72°C for 2 minutes.	455bp
c-Myc	95°C for 2 minutes, 20 to 23 cycles of [95°C for 30 seconds, 55.3°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.	185bp

Table 2.2: PCR cycling parameters and product sizes (continued)

Transcript	Cycling parameters	Product size
cyclin D2	95°C for 3 minutes, 23 cycles of [95°C for 30 seconds, 55.3°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.	240bp
nucleolin	95°C for 3 minutes, 35 cycles of [95°C for 1 minute, 65°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.	105bp

2.5 Preparation of whole cell extracts

All steps were performed on ice or at 4°C. Buffers, reagents and plastic-ware were kept chilled.

2.5.1 Preparation of extracts for *in vitro* transcription assays

Extracts for *in vitro* transcription assays (IVTs) were prepared from cardiomyocytes grown on 100mm tissue culture dishes. Cells were placed on ice then washed twice in phosphate-buffered saline (PBS; 170 mM NaCl, 3.4 mM KCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2). Cells were then scraped into PBS (5ml/dish), and collected in 50ml Falcon tubes. Cells were pelleted by slow centrifugation at 500g (in a Sorvall RT 6000 D), then the PBS was discarded. A small amount of fresh PBS (approximately 1ml) was then added to the cells to aid transfer into sterile microfuge tubes. This PBS was then also removed following centrifugation (in an Eppendorf Centrifuge 5415R) at 12000g for 1 minute. The remaining cell pellets were gently resuspended in freshly made microextraction buffer (450 mM NaCl, 50 mM NaF, 20 mM HEPES pH 7.8,

25% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM EDTA, 40 μg/ml bestatin, 1 μg/ml trypsin inhibitor, 0.7 μg/ml pepstatin, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin). The volume of microextraction buffer used was equivalent to the volume of the cell pellet. Following resuspension, samples were immediately snap-frozen on dry ice, then thawed at 30°C. When just thawed, samples were again returned to dry ice. This freeze-thaw procedure was performed a total of 3 times to ensure optimal cell lysis. Following the final thaw, cell debris was collected by centrifugation at 12000g for 10 minutes. The supernatant was promptly aliquoted, then snap-frozen. Samples were stored at -80°C. Aliquots were not used more than twice for IVTs.

2.5.2 Preparation of extracts for polyacrylamide gel electrophoresis

Extracts for polyacrylamide gel electrophoresis were prepared from cardiomyocytes grown on 6-well plates. Cells were placed on ice then washed twice in PBS. Cells were then scraped directly into cell lysis buffer (20 mM HEPES (pH 7.8), 150 mM NaCl, 25% glycerol, 50 mM NaF, 0.2 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT, 0.5 μg/ml leupeptin, 1.0 μg/ml trypsin inhibitor, 0.5 μg/ml aprotinin and 40 μg/ml bestatin) and transferred to sterile microfuge tubes. 100 μl of buffer was used per well. The cell lysates were incubated on ice for 15 minutes, and then passed through a 26-guage needle three times. Cell debris was collected by centrifugation at 16000g for 10 minutes, and the supernatants were aliquoted, snap-frozen on dry ice, then stored at –80°C.

2.5.3 Determination of protein concentrations

The protein concentrations of whole cell extracts were determined using Bradford's reagent (BioRad) diluted 1 in 5 with distilled H_2O. The colour change produced upon mixing this reagent with protein can be quantified by measuring absorbance at 595 nm, and is directly proportional to the concentration of protein in the sample. For each experiment, a standard curve was constructed by measuring the absorbance (using a spectrophotometer) of 1, 2, 4, 6, 8, 10 and 12 μg of BSA in 1ml of Bradford's reagent. Whole cell extracts were diluted 1 in 10 with microextraction buffer, then 10 μl added to 1 ml of reagent. Absorbance readings at 595 nm were performed in triplicate, and the protein concentration of each sample determined from the standard curve.

2.6 Storage, propagation and preparation of plasmid DNA

2.6.1 Transformation of competent cells

For plasmid storage and propagation, *E.coli* XL-1 blue supercompetent cells (Stratagene) were transformed. These cells were stored at –80°C and were thawed on ice prior to use, to prevent loss of transformation efficiency. 10-20 ng of plasmid DNA was added to 50 μl of thawed cells and mixed gently. The mixture was incubated on ice for 30 minutes, with occasional gentle agitation. Following this time, cells were heat-shocked for exactly 45 seconds at 42°C, then transferred to ice for a further 2 minutes. 450 μl of SOC medium (LB broth, 0.04% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$), which had been pre-heated to 42°C, was then added and cells were incubated at 37°C for 1 hour on an orbital

shaker (225-250 rpm). Subsequently, 150 µl of the transformation mixture was plated on LB-agar (2% LB, 2% agar) containing 50 µg/ml of the selective antibiotic ampicillin, and then incubated at 37°C overnight to allow colony formation.

2.6.2 Preparation of plasmid DNA

An isolated bacterial colony was selected from a streaked LB-agar plate, and used to inoculate 4 ml of LB medium containing 50 µg/ml ampicillin. This mini-culture was incubated at 37°C for approximately 6 hours on an orbital shaker (300rpm), then used to inoculate 250 ml of LB medium containing 50 µg/ml ampicillin. This larger culture was incubated overnight under the same conditions. The following day, plasmid DNA was purified from the bacterial cells using the QIAGEN Plasmid Maxi Kit, according to the manufacturer's instructions.

Bacterial cells were harvested by centrifugation (in Sigma Laboratory Centrifuge 4K15) at 6000g for 15 minutes at 4°C, then resuspended in 10 ml of Buffer P1 (500 mM Tris pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Cell lysis was performed by adding 10 ml of Buffer P2 (200 mM NaOH, 0.1% SDS). This reaction was allowed to proceed at room temperature for 5 minutes before lysates were neutralised by adding 10 ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5). Addition of Buffer P3 caused the precipitation of potassium dodecyl sulphate, SDS-denatured proteins, chromosomal DNA and cell debris. Precipitation was enhanced by a 20 minute incubation on ice. Plasmid DNA is circular, and therefore renatured correctly and remained in solution.

Centrifugation at 20000g for 30 minutes was performed (at 4°C) to separate precipitated debris from soluble material. Following this centrifugation, the supernatant containing plasmid DNA was promptly removed and applied to a QIAGEN-tip 500, pre-equilibrated with 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). Plasmid DNA binds tightly to QIAGEN-tip resin, while the remainder of the supernatant passes through by gravity flow. The resin was then washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol), before eluting the purified plasmid DNA into a Falcon tube with 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris pH 8.5, 15% isopropanol). DNA was precipitated with 10.5 ml of isopropanol. The sample was then centrifuged at 15000g for 30 minutes at 4°C. Following this, the supernatant was carefully decanted out leaving the pelleted plasmid DNA, which was then washed with 70% ethanol and recentrifuged. The pellet was air-dried for approximately 10 minutes, then resuspended in an appropriate volume of sterile distilled H_2O. DNA concentration was determined by measuring absorbance at 260 nm, and using the following calculation: DNA concentration (μg/ml) = absorbance at 260 nm x 50 x dilution factor. All plasmid DNA stocks were stored at -20°C.

2.7 Pol III *in vitro* transcription assay

IVTs were performed using 15 to 20 μg of cell extract (prepared as described in section 2.5.1) and 250 ng of plasmid DNA (prepared as described in section 2.6) containing the class III templates $tRNA^{Leu}$ (pLeu: a 240bp EcoRI-HindIII fragment of human genomic DNA carrying a $tRNA^{Leu}$ gene subcloned into

pAT153), VAI (pVAI: a 221bp SalI-BalI fragment of adenovirus 2 DNA containing the VAI gene subcloned into pUC18), B2 (pTB14: a 0.2kb BglII fragment containing the B2 gene from TP2 subcloned into pUC18) or 5S rRNA (pMO5S1.1: a 436bp SacI-NheI fragment of mouse DNA cloned into pBluescript KS-). Transcription was carried out at 30°C for 1 hour in a total volume of 25 µl containing 12 mM HEPES pH 7.9, 60 mM KCl, 7.2 mM $MgCl_2$, 0.28 mM EDTA, 1.2 mM DTT, 10% glycerol, 1 mM creatine phosphate, 0.5 mM of each rATP, rCTP and rGTP, and 10 µCi of [α-^{32}P]UTP (Amersham). Transcription was stopped by the addition of 250 µl of 1 M ammonium acetate/0.1% SDS containing 20 µg of yeast tRNA (which stabilises the newly synthesised RNA in the samples). Phenol-chloroform extraction was then performed, to remove protein and DNA, by adding 250 µl of a 25:24:1 ratio solution of phenol/chloroform/isoamyl alcohol to each sample. Samples were mixed thoroughly by vortexing, then centrifuged at 13000g for 5 minutes. 200 µl of the resulting upper aqueous layer was then transferred to a fresh microfuge tube containing 750 µl of ethanol. The samples were mixed by repeated inversion, and left at -20°C overnight to precipitate RNA. The following day, samples were centrifuged at 13000g for 30 minutes to pellet the precipitated RNA. The supernatant was carefully removed and discarded, then pellets were washed using 750 µl of 70% ethanol (prepared using DEPC-treated H_2O) and re-centrifuged at 13000g for 5 minutes. Again, the supernatant was discarded. RNA pellets were dried at 50°C for 5 minutes. Once dry, 4 µl of formamide loading buffer was added to each sample. Samples were then vortexed for 30 minutes to ensure complete resuspension of the RNA, and heated at 95°C for 2 minutes.

Electrophoresis and autoradiography of radiolabelled transcripts was performed as described in section 2.4.2 for PCR products.

2.8 Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detection by Western blotting

2.8.1 SDS-PAGE

Whole cell lysates (prepared as described in section 2.5.2) containing 75 μg of protein were resolved by denaturing SDS-PAGE on 7.8% (unless otherwise indicated) polyacrylamide minigels (375 mM Tris pH 8.8, 0.1% SDS), with 4% polyacrylamide stacking gels (125 mM Tris pH 6.8, 0.1% SDS). Prior to loading, samples were boiled for 4 minutes in 1 X protein sample buffer (62.5 mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue). Electrophoresis was performed in 1 X SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris pH 8.3) at an initial voltage of 80V. Once the bromophenol blue die had moved through the stacking gel and reached the resolving gel, the voltage was increased to 140V and electrophoresis was continued for 60 to 90 minutes.

2.8.2 Western blot analysis

Following resolution by SDS-PAGE, proteins were transferred to a PVDF membrane (Amersham) using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 X transfer buffer (76.8 mM

glycine, 10 mM Tris pH 8.3, 16.5% methanol) at 50V for 2 hours at room temperature. Membranes were then blocked in milk buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20, 5% skimmed milk powder (Marvel)) for 1 hour at room temperature, prior to incubation overnight at 4°C with the relevant primary antibody diluted in milk buffer. The primary antibodies utilised for Western blotting are listed in Table 2.3. The following day, membranes were washed 3 times for 5 minutes in milk buffer, to remove excess primary antibody. Subsequently, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako), diluted 1 in 1000 in milk buffer, for 1 hour at room temperature. Excess secondary antibody was then removed by three 10 minute washes in fresh milk buffer. Following a final 5 minute wash in 1 X TBS (2.5 mM Tris pH 7.6, 15 mM NaCl), the bound antibodies were detected using the enhanced chemiluminescence (ECL) method (Amersham), as directed by the manufacturer.

Table 2.3: Primary antibodies used for Western blot analysis

Protein Detected	Antibody	Supplier	Dilution in milk buffer
Brf1	128	In house	1:1000
Bdp1	2663	In house	1:1000
TBP	58C9	Santa Cruz Biotechnology	1:1000
TFIIIC110	4286	In house	1:1000
TFIIIC220	Ab 7	In house	1:1000
Active ERK1 and 2	E10	Cell Signalling Technology	1:1000
Total ERK1 and 2	9102	Cell Signalling Technology	1:1000
c-Myc	9E10	Santa Cruz Biotechnology	1:500
c-Myc	C8	Santa Cruz Biotechnology	1:500
RB phosphorylated on threonine 826	44-576	Biosource International	1:500
RB phosphorylated on serine 780	9307	Cell Signalling Technology	1:500
RB phosphorylated on serine 795	9301	Cell Signalling Technology	1:500
Total RB	C15	Santa Cruz Biotechnology	1:1000
Total RB	G3-245	BD PharMingen Biosciences	1:500
Cyclin D1	72-13G	Santa Cruz Biotechnology	1:1000
$p16^{INK4a}$	H-156	Santa Cruz Biotechnology	1:1000
Haemagglutinin (HA)	F-7	Santa Cruz Biotechnology	1:1000
Actin	C11	Santa Cruz Biotechnology	1:1000

2.9 Coulter Z2 analysis of cardiomyocyte volume and number

Prior to analysis in the Coulter Z2 particle count and size analyser (Beckman Coulter), cardiomyocytes were dissociated from dishes using trypsin, then resuspended in DMEM/M199 (4:1) supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin. 10 ml of this medium was used per 100mm tissue culture dish. 0.4 ml of this cell suspension was then added to a vial containing 10 ml of Isoton II electrolyte solution (filtered PBS solution, Beckman Coulter), and analysed using the Coulter Z2 with a 100 μm aperture. This aperture was pre-calibrated using spherical latex beads with 10 μm diameter (Beckman Coulter), according to the manufacturer's instructions. For each analysis, the Coulter Z2 measured 0.5 ml of the electrolyte cell suspension. The Z2 determines cell number and volume by measuring the frequency and size of electrical pulses generated as each cell enters the aperture. These changes in electrical current are collated by the Z2, and displayed as a histogram of cell number versus volume. From this, the number and mean volume of cells above a certain threshold could be obtained. This lower threshold was set to exclude any cell debris and dust particles from the analysis, and was in the range of approximately 400 to 500fl. Two 100mm dishes were analysed per condition for each experiment, and each sample was measured three times. The aperture was flushed with Isoton II before and after each analysis.

2.10 DNA synthesis assay

For measurements of DNA synthesis, cardiomyocytes were plated on 24-well tissue culture dishes. Cardiomyocytes were treated accordingly (3 wells per condition), depending on the experiment, and [^{3}H]thymidine (0.1 μCi/ml) was added 16 hours prior to harvesting. Cells were then transferred to ice and washed twice in ice-cold PBS, three times in 5% trichloroacetic acid (TCA), and twice in ethanol. Samples were solubilized in 0.3 M NaOH and transferred to scintillation vials, to which 3ml of scintillation fluid (Fisher Scientific) was added. The incorporation of [^{3}H]thymidine into DNA was measured by liquid scintillation counting.

2.11 Protein synthesis assay

Cardiomyocytes were grown on 24-well plates, and treated as required depending on the experiment. Three wells were used for each different condition. One hour prior to harvesting, 5 μCi of [^{35}S]methionine/cysteine (Amersham) was added per ml of medium to label cellular proteins. Cells were then washed twice with ice-cold PBS on ice, and scraped into 50 μl of cell lysis buffer (as defined in section 2.5.2). 10 μl of each extract was spotted onto a 2 x 2cm piece of 3MM Whatmann paper, in duplicate. The paper was dropped into 5% TCA containing a small amount of 'cold' methionine (Sigma), and boiled for 2 minutes. The TCA was discarded and replaced with fresh TCA/methionine before boiling again for 2 minutes. Filters were then rinsed once in cold TCA, and once in ethanol. Filters were air-dried, then transferred to scintillation vials, and 3 ml of scintillation

fluid was added. Incorporation of radiolabelled amino acids was determined by liquid scintillation counting.

2.12 Infection of cardiomyocytes with replication-deficient recombinant adenoviruses

Cardiomyocytes were cultured in 6-well plates for 48 hours after isolation, before infections were performed. Infections were carried out in a class II hood, and viral waste and contaminated equipment was discarded according to institutional guidelines. All viruses were stored in small aliquots at -80°C, and thawed at room temperature before use. Aliquots were not freeze-thawed more than twice.

2.12.1 MEK1-expressing adenovirus

Dr JD Molkentin (Departments of Medicine and Cell Biology, Einstein College of Medicine, The Bronx, New York, USA) provided equivalent titres of adenoviruses expressing β-galactosidase (β-gal) alone (control) or β-gal and constitutively active MEK1 (CAMEK). Viruses were shipped on dry ice. Prior to infection, cardiomyocyte medium was replaced with fresh maintenance medium (2 ml/well). Subsequently, control or CAMEK-expressing viruses were added (10, 25 or 40 μl per well, as indicated for each experiment), and infection was allowed to proceed for 16 hours at 37°C in a humidified, 5% CO_2 incubator. Following this time, medium was aspirated off and replaced with serum-free medium (3 ml/well). Cells were incubated for a further 48 hours prior to harvesting for RNA and protein, as described in sections 2.2.1 and 2.5.2,

respectively. Efficient expression of CAMEK was confirmed by Western blotting for active ERK.

2.12.2 $p16^{INK4a}$-expressing adenovirus

Dr S Mittnacht (Centre for Molecular and Cell Biology, The Institute of Cancer Research, Chester Beatty Laboratories, London, UK) supplied equivalent titres of adenoviruses expressing green fluorescent protein (GFP) alone (control) or GFP and $p16^{INK4a}$. Again, viruses were transported on dry ice. Infection was carried out for 16 hours in 2 ml/well of fresh maintenance medium using 25 µl of control or $p16^{INK4a}$-expressing adenoviruses. Both viruses infected approximately 90% of cells, as confirmed using a fluorescent microscope (Axiovert 25, Carl Zeiss). Following 16 hours of exposure to virus, the maintenance medium was replaced with fresh (3 ml/well). 32 hours later, cells were changed into serum-free medium or medium containing 10% FCS. Cells were harvested after 16 hours for RNA and protein, as described in sections 2.2.1 and 2.5.2, respectively. $p16^{INK4a}$ expression was confirmed by Western blotting.

2.12.3 HA-tagged Brf1 (HA-Brf1)-expressing adenovirus

Adenoviruses expressing GFP alone (control) or GFP and HA-Brf1 were generated, amplified and titred by Dr F Cairns (Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, UK). Cells were infected by adding 1.5×10^6 plaque forming units of control or HA-Brf1-expressing virus to wells containing 2 ml of fresh serum-free medium (giving a multiplicity of infection of 2). Cells were incubated for 4 hours, then serum-free medium was replaced with fresh maintenance medium. 24

hours later, cells were serum-starved or stimulated with 10% FCS for 16 hours. Cells were then harvested for RNA and protein, as described in sections 2.2.1 and 2.5.2, respectively. Both viruses infected approximately 75% of cells, as determined using a fluorescent microscope. HA-Brf1 expression was confirmed by Western blotting.

2.13 Chromatin immunoprecipitation (ChIP) assay

Cardiomyocytes were grown on 100mm tissue culture dishes for ChIP assays. Ten dishes were used per condition (serum-free or stimulated with 10% FCS). Medium was aspirated from the cells, then cells were washed once in pre-warmed 37°C PBS. To crosslink cellular DNA and proteins, 7ml of pre-warmed PBS containing 1% formaldehyde was added to each dish. Crosslinking was allowed to proceed for 10 minutes at 37°C, then glycine was added at a final concentration of 0.125 M to stop the crosslinking, and plates were transferred to ice. Cells were then harvested in the PBS/formaldehyde/glycine solution, and transferred to Falcon tubes. Tubes were centrifuged (in a Sorvall RT 6000 D) at 500g for 5 minutes at 4°C, then cells were washed twice by resuspension in ice-cold PBS, followed by centrifugation at 500g for 5 minutes. Following removal of supernatant after the final wash, cells were resuspended in 40 ml of high salt buffer (0.5% NP-40/PBS, 1 M NaCl) and incubated on ice for 30 minutes. Cells were then centrifuged at 500g for 5 minutes, and washed once in 0.5% NP-40/PBS. Subsequently, hypotonic disruption was performed by resuspending cells in 40 ml of low salt buffer (0.1% NP-40, 10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl) and incubating on ice for 30 minutes. Following this, samples were

centrifuged at 500g for 5 minutes at 4°C, and the resulting pellets resuspended in 1 ml of low salt buffer. To obtain nuclei, samples were then passed through a 26-guage needle 3 times. The samples were re-centrifuged as before, but this time the pellets were resuspended in 2.7 ml of low salt buffer, then lysed with 300 µl of 20% sarkosyl. Subsequently, lysed nuclei were transferred to a sucrose cushion (40 ml low salt buffer/100 mM sucrose) and centrifuged at 4000g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 3 ml of TE (10 mM Tris pH 8.0, 1 mM EDTA). This 3 ml was then applied to a second sucrose cushion and the centrifugation process repeated. The final pellet containing genomic DNA was resuspended in 2 ml of TE, and then the DNA was sheared into smaller fragments (1kb on average) by sonication (Branson Sonifier 250, 10 X for 10 second intervals, 30% duty cycle). 0.2 ml of 11 X NET buffer (1.65 M NaCl, 5.5 mM EDTA, 5.5% NP-40, 550 mM Tris pH 7.4) was added to 2ml of sonicated material, then this was transferred to microfuge tubes for centrifugation at 13000g for 5 minutes. The supernatants were then aliquoted evenly into microfuge tubes. Each aliquot was incubated in the presence of 5 µg (25 µl) of an appropriate antibody overnight at 4°C on a rotating wheel. Antibodies used for ChIP analysis are listed in Table 2.4. As a negative control, one aliquot was incubated in the absence of antibody. Also, 10% of the aliquot volume was retained for use as an input control.

The following day, Protein-A-Sepharose beads (Amersham) were added for a further 2 hours of incubation, and then recovered on Polyprep columns (BioRad). Columns were washed twice in 10 ml of RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), twice in 10 ml of LiCl

buffer (10 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA pH 8.0) and twice in TE. These washes were performed at 4°C. Beads were then transferred to 1.5 ml microfuge tubes and immunoprecipitated material eluted by incubating the beads with 100 µl of 1% SDS/TE on a rotating wheel for 10 minutes at room temperature. This was performed twice, and each time the supernatant was collected following centrifugation at 13000g for 1 minute. The pooled supernatants (and input DNA made up to a total volume of 400 µl with 1% SDS/TE) were incubated overnight at 42°C with 0.125 mg/ml Proteinase K to degrade the antibodies and any other proteins present. Subsequently, genomic DNA was extracted twice using 400 µl phenol/chloroform/isoamylalcohol (25:24:1). Ethanol precipitation was performed overnight at -20°C. The immunoprecipitated DNA was then resuspended in 40 µl TE, and quantified by PCR. 1 µl of immunoprecipitated or input DNA was used per PCR reaction following the protocol outlined in section 2.4.2. PCR primers and cycling parameters are indicated in Tables 2.1 and 2.2.

Table 2.4 Antibodies used for ChIP assays

Protein recognised	Antibody	Supplier
Brf1	128	In house
Bdp1	2663	In house
Largest pol III subunit (RPC 155)	1900	In house
TFIIIC110	4286	In house
TFIIIC220	Ab 7	In house
c-Myc	9E10	Santa Cruz Biotechnology
c-Myc	N262	Santa Cruz Biotechnology
TFIIB	C18	Santa Cruz Biotechnology

2.14 Quantification and statistical analysis of data

Data were quantified by densitometry using TotalLab (photoretix) version 1.11. Statistical analysis was performed using a Student's t-Test (Microsoft Excel; two-tailed distribution, unequal variance). A probability (p) value of less than 0.05 was taken as a statistically significant difference between two groups.

CHAPTER 3
RNA Polymerase III transcription is activated during cardiomyocyte hypertrophy

3.1 Introduction

The mammalian heart is primarily composed of cardiomyocytes. As outlined in Chapter 1, these cells become terminally differentiated and largely lose the ability to proliferate shortly after birth (Brooks et al, 1998; Leu et al, 2001; Li et al, 1997; Soonpaa et al, 1996). As a result, myocardial hypertrophy, characterised by an increase in myocyte volume in the absence of cell division, is the major determinant of heart mass during post-natal development (Bugaisky et al, 1992; Hannan and Rothblum, 1995; Leu et al, 2001; Zak, 1974). In addition to this developmental role, cardiomyocyte hypertrophy can also occur in the adult heart under various circumstances. For instance, exercise training in athletes can lead to a modest increase in heart size through cardiomyocyte hypertrophy (Frey and Olson, 2002; Hunter and Chien, 1999; Linzbach, 1960; Selvetella et al, 2004). This beneficial adaptation increases the contractile capacity of the heart, allowing it to cope with the increased demands associated with intensive exercise. However, in industrialised countries, hypertrophy in adults is most commonly associated with cardiac disease, and is a well-established risk factor for cardiovascular mortality (Harjai et al, 1999; Kannel, 1990; Levy et al, 1990; Vakili et al, 2001). Therefore, the causes and molecular events underlying this pathological hypertrophic response have been the subject of intensive investigation.

Cardiomyocyte hypertrophy develops during most cardiovascular disorders as a result of chronic mechanical and/or neuro-hormonal abnormalities. For instance, increased mechanical loading has been well documented to induce hypertrophy in the heart and in cultured cardiomyocytes (Komuro et al, 1990; Morgan and Baker, 1991; Sadoshima and Izumo, 1997; Sadoshima et al, 1992). In the physiological setting, this results from haemodynamic alterations, usually caused by high blood pressure or defects in contractile efficiency. Ion channels, integrins and receptor tyrosine kinases have all been implicated in detecting this mechanical load and converting it to intracellular signals (Ross, 2004; Sadoshima and Izumo, 1997; Sussman et al, 2002; Yamazaki et al, 1998). Although mechanical stress can induce hypertrophic growth directly (Komuro et al, 1990; Komuro et al, 1991; Yamazaki et al, 1998), the release of autocrine/paracrine factors may contribute to the development of this load-induced hypertrophy (Sadoshima and Izumo, 1993a; Sadoshima and Izumo, 1997; Yamazaki et al, 1998). In particular, the peptide hormones angiotensin II and ET-1 are thought to play important roles in mediating the hypertrophic response to increased workload (Ito et al, 1993; Ito et al, 1994; Rockman et al, 1994; Sadoshima et al, 1993).

Hormonal signals are also capable of stimulating cardiomyocyte hypertrophy independently of load (Morgan and Baker, 1991; Simpson et al, 1982). The best characterised of these factors include ET-1, angiotensin II, and α_1-adrenergic agonists, such as PE (Chien et al, 1991; Lee et al, 1988; Morgan and Baker, 1991; Sadoshima and Izumo, 1993b; Shubeita et al, 1990). These bind to specific, seven-transmembrane G-protein coupled receptors found on the surface

of cardiomyocytes, which couple to the intracellular second messenger G_q. The importance of G_q-coupled receptors in mediating the hypertrophic response has been demonstrated by numerous studies (reviewed in Dorn and Brown, 1999; and Molkentin and Dorn, 2001). For example, transgenic mice overexpressing these receptors, or G_q itself, in the heart, display myocardial hypertrophy, whereas cardiac-specific conditional inactivation of G_q ablates pressure overload-induced hypertrophy in adult mice (Akhter et al, 1998; D'Angelo et al, 1997; Frey and Olson, 2002; Milano et al, 1994; Paradis et al, 2000). Other extracellular stimuli, which bind a range of cell-surface receptor types, have been reported to induce the hypertrophic growth of cardiomyocytes, including fibroblast growth factors, insulin-like growth factor 1, prostaglandin $F_{2\alpha}$ and cytokines such as cardiotrophin-1 (Sugden, 1999).

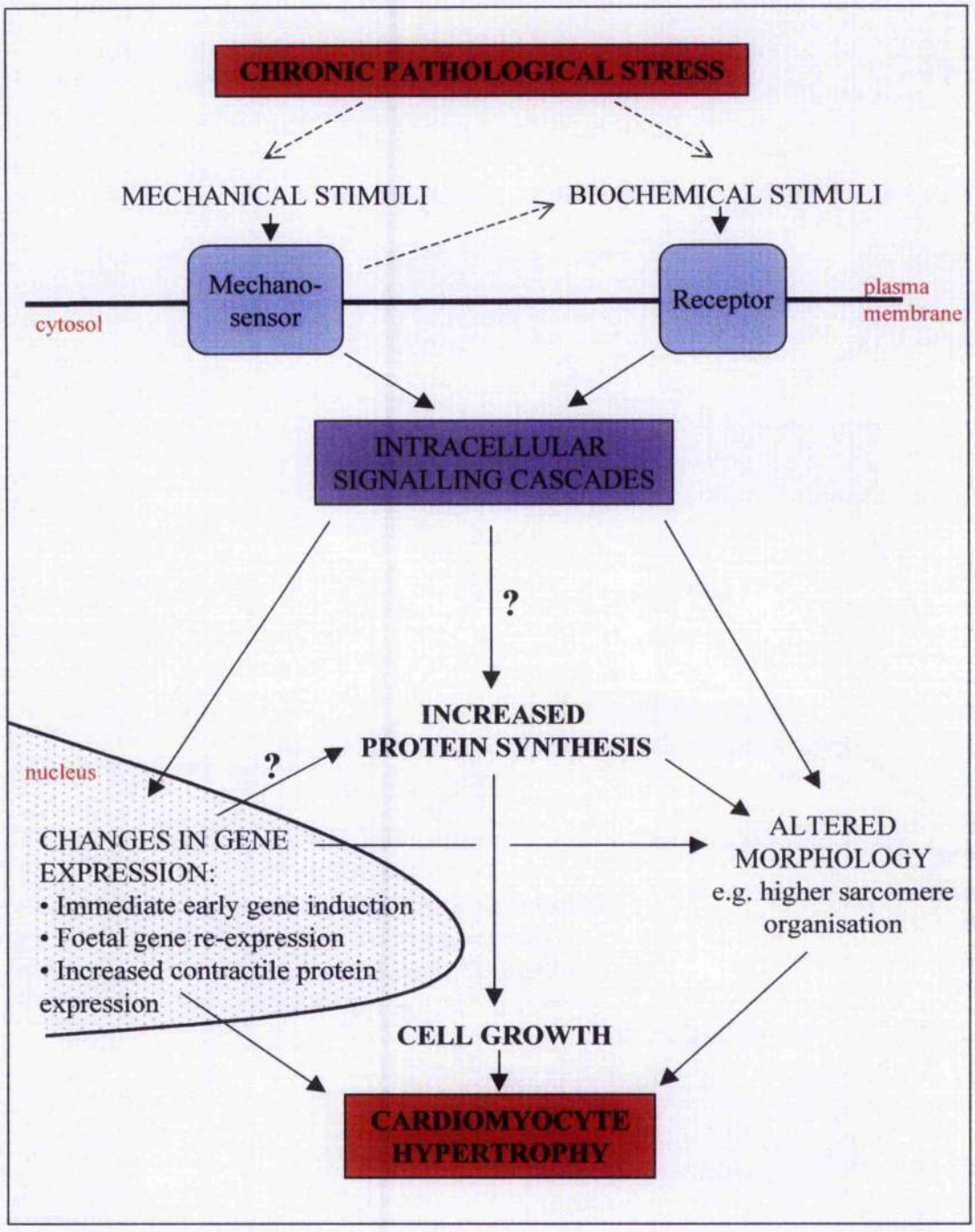
Regardless of the nature of the initiating trigger, detection of a hypertrophic stimulus at the cell surface is followed by the activation of a wide array of intracellular signalling cascades. Impressive advances have been made towards characterising this extensive, hypertrophy-associated signalling network, now known to encompass a variety of signal transducing molecules, including the small G-protein ras, protein kinase C (PKC), various members of the mitogen-activated protein (MAP) kinase family, phosphatidylinositol 3-kinase (PI3K) and the calcium-regulated signalling proteins calmodulin and calcineurin (reviewed in Hoshijima et al, 2004, and Molkentin and Dorn, 2001). The activation of these pathways culminates with hypertrophic cardiac growth, which is largely due to an increase in protein content per individual cardiac myocyte, through increased rates of protein synthesis, in the absence of cell division (Chien et al, 1991;

Morgan et al, 1987). In addition, other cardinal features specific to the maladaptive hypertrophic response accompany this cell growth, including characteristic morphological changes, such as increased sarcomere organisation, and alterations in gene expression. The latter includes the induction of immediate early genes (e.g c-fos, c-jun and c-myc), the re-expression of certain foetal genes (such as ANF, β–myosin heavy chain and skeletal muscle α-actin), and the upregulation of constitutively expressed contractile proteins (Chien et al, 1991). This programme of molecular events induced during hypertrophy is summarised in Figure 3.1.

One of the current goals of cardiovascular research is to understand how these signalling pathways influence downstream events, ultimately leading to the induction of the features of hypertrophy described above. The majority of studies in this regard, have so far focused on elucidating the processes involved in mediating characteristic changes in cardiomyocyte gene expression, such as the re-expression of foetal genes. This has revealed specific roles for MAP kinase and calcineurin pathways, which regulate gene expression by altering the activity of various transcription factors (reviewed in Hoshijima et al, 2004). However, less emphasis has been placed on determining the molecular mechanisms by which hypertrophy-inducing signalling cascades increase myocyte protein synthesis, a fundamental feature of hypertrophic growth.

A cell has the potential to increase its rate of protein synthesis through various means. Mechanisms proposed to facilitate the increased rates of protein synthesis required for the hypertrophic growth of cardiomyocytes, include enhanced

Figure 3.1: Summary of the molecular events induced by hypertrophic stimulation of cardiomyocytes. A range of hormonal and mechanical stimuli activate a wide array of receptors expressed on the surface of cardiomyocytes. This leads to the activation of numerous intracellular signalling cascades, which in turn cause an alteration in the cardiomyocyte gene expression programme, an increase in the overall rate of cardiomyocyte protein synthesis, and changes in cellular morphology. However, the precise mechanisms through which these signalling pathways regulate protein synthesis, which underlies cell growth, remain unclear, as indicated by '?'.


CHRONIC PATHOLOGICAL STRESS
MECHANICAL STIMULI
BIOCHEMICAL STIMULI
Mechano-sensor
Receptor
cytosol
plasma membrane
INTRACELLULAR SIGNALLING CASCADES
?
INCREASED PROTEIN SYNTHESIS
nucleus
?
CHANGES IN GENE EXPRESSION:
• Immediate early gene induction
• Foetal gene re-expression
• Increased contractile protein expression
ALTERED MORPHOLOGY
e.g. higher sarcomere organisation
CELL GROWTH
CARDIOMYOCYTE HYPERTROPHY

translational efficiency and capacity (reviewed in Hannan et al, 2003). Translational efficiency is controlled at the levels of peptide chain initiation and elongation, which in turn are regulated by specialised translation factors (Proud, 2004). Several lines of evidence suggest that the activities of certain translation initiation and elongation factors are regulated in response to hypertrophic stimulation of cardiomyocytes (Everett et al, 2001; Hardt et al, 2004; Nagatomo et al, 1999; Saghir et al, 2001; Wada et al, 1996; Wang and Proud, 2002a). Although initially this may contribute to increased rates of protein synthesis (Hannan et al, 2003; Nagatomo et al, 1999), it is widely accepted that this is not sufficient to account for sustained hypertrophic growth, and it has been shown that, in terminally differentiated cardiomyocytes, cell growth accompanies an increase in translational capacity (Chua et al, 1987; McDermott and Morgan, 1989; Morgan et al, 1987; Siehl et al, 1985).

Translational capacity is determined by the availability of components of the protein synthetic machinery, including ribosomes, tRNAs and translation factors. Transcription by pol I, which synthesises the large rRNAs, is increased during hypertrophy (Hannan et al, 1995; Hannan et al, 1996a; Luyken et al, 1996; McDermott et al, 1989; McDermott et al, 1991), and this is essential for the induction of cardiomyocyte growth (Brandenburger et al, 2001). Pol III synthesises 5S rRNA, which is found in the ribosome in equimolar amounts with the other, pol I-produced rRNAs (Wool, 1979). Furthermore, pol III generates other essential components of the protein synthetic machinery (as detailed in Chapter 1), including tRNAs. Because of the crucial involvement of class III gene products in various aspects of cellular metabolism, the rate of pol III

transcription is tightly correlated with the growth state of cells (reviewed in Chapter 1). For example, several studies in various eukaryotic systems have demonstrated that pol III transcription is subject to stringent control during many fundamental cellular processes, which require changes in the rate of protein synthesis, such as during the cell cycle and differentiation, and in response to nutrients and mitogens (Leresche et al, 1996; Scott et al, 2001; Sethy et al, 1995; Tower and Sollner-Webb, 1988; White et al, 1989; White et al, 1995a, b). Therefore, it is highly likely that, as with pol I transcription, pol III transcription will also be activated during myocardial hypertrophy. However, pol III transcriptional regulation has not yet been thoroughly investigated in cardiac muscle cells. This is an important issue to address, in order to enhance understanding of the processes facilitating hypertrophic growth. The experiments described in this chapter aimed to determine whether increased pol III transcription is a feature of the hypertrophic response, through the use of well-defined model systems.

3.2 Results

3.2.1 Characterisation of cultured cardiomyocytes

The current understanding of the hypertrophic growth response has been greatly facilitated by the development and characterisation of cultured cardiomyocyte cell models by several investigators (reviewed in Chien et al, 1991). Most commonly, these cells are prepared directly from neonatal rat hearts. The suitability of these primary cells for studying the molecular events involved in myocardial growth has been well documented. Although prepared from neonates, numerous studies have demonstrated that exposure of these cells to various agents induces many features of hypertrophy seen in adult cardiomyocytes in the heart (Bishopric et al, 1987; Chien et al, 1991; Knowlton et al, 1991; Lee et al, 1988; Meidell et al, 1986; Shubeita et al, 1990; Simpson et al, 1982; Starsken et al, 1986; Waspe et al, 1990). For example, ET-1, PE and also FCS induce morphological, structural, biochemical and transcriptional changes in these cultured cardiomyocytes characteristic of those induced in the myocardium.

In this study, cardiomyocytes were prepared from the hearts of neonatal Sprague Dawley rats, using previously described methods with minor modifications (as described in Chapter 2). Following dissociation of the various types of cardiac cell from rat heart tissue, procedures that selectively enrich for cardiomyocytes were employed. This resulted in spontaneously contracting cultures of cardiomyocytes of greater than 90% purity, as determined by microscopic and immunofluorescence analysis (data not shown), in accordance with what has been reported in the literature (Boluyt et al, 1997; Flink et al, 1998; Poolman and Brooks, 1998; Sadoshima et al, 1997; Starsken et al, 1986; von Harsdorf et al,

1999). To further ensure that the myocyte isolation procedure used in this study produced a reliable model system, a series of experiments were performed to characterise the hypertrophic growth response of these cells, before examining the regulation of pol III transcription.

As discussed previously, several criteria typify the hypertrophic growth of cardiomyocytes. One of the cardinal features of this response is an increase in the rate of protein synthesis (Chien et al, 1991; Morgan et al, 1987). Because this occurs in the absence of cell division, DNA synthesis is not normally induced. Cultured cardiomyocytes were serum-starved for 24 hours, then treated for 16 hours with classic hypertrophic stimuli, including FCS, ET-1 and PE. Control cells were maintained in serum-free media. The effects of these treatments on protein synthesis and DNA synthesis were assessed by measuring the incorporation of radiolabelled cysteine and methionine, or thymidine, respectively. As revealed in Figure 3.2, each of these stimuli caused a significant increase in the rate of protein synthesis compared to control cells, within the range previously reported in the literature (Boluyt, et al, 1997; Bueno et al, 2000; Nozato et al, 2001; Pham et al, 2001; Sano et al, 2002; Tamamori et al, 1998). DNA synthesis was not induced, as expected (Sadoshima et al, 1997; Tamamori et al, 1998).

Accelerated protein synthesis, and the resulting accumulation of protein, underlies the increase in cardiomyocyte size characteristic of hypertrophy. To confirm that increased protein synthesis was accompanied by cell growth in the present study, cardiomyocyte volume was measured using a Z2 Coulter counter.

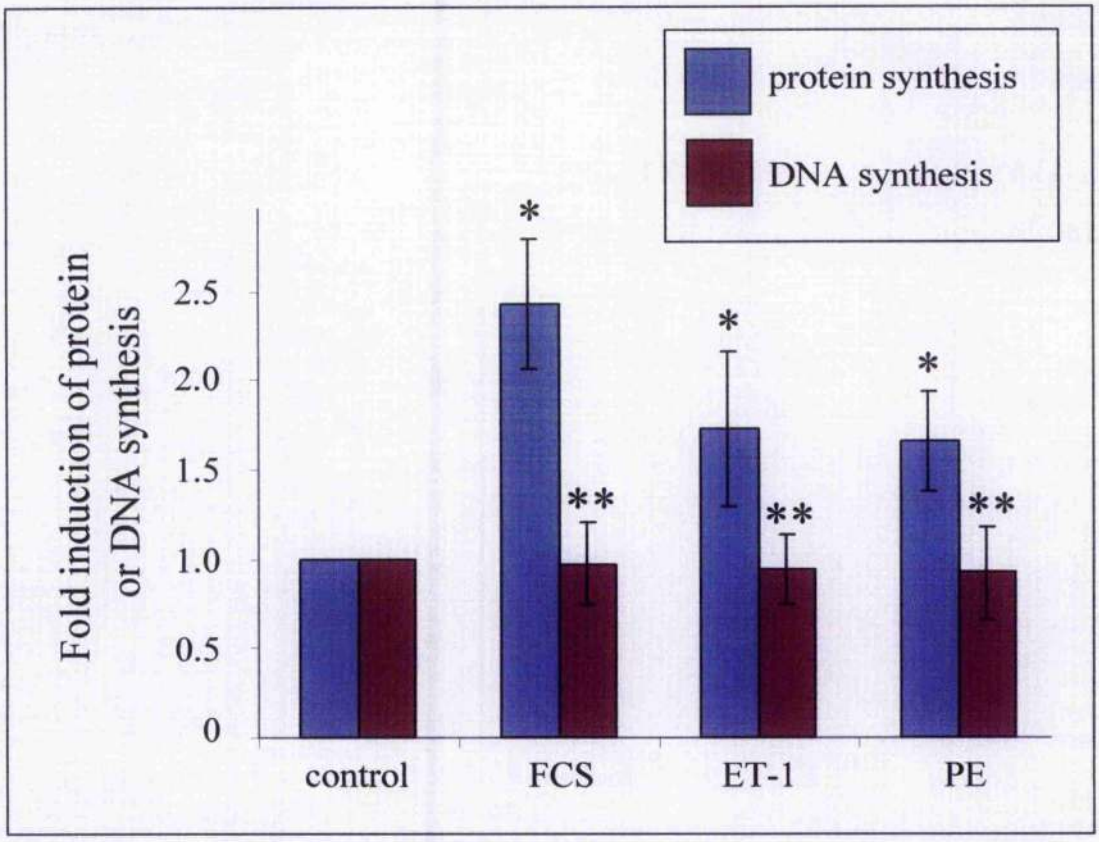


Figure 3.2: Various stimuli induce protein synthesis, but not DNA synthesis, in primary cardiomyocyte cultures. Cultured cardiomyocytes were serum-starved for 24 hours, then either maintained in serum-free media for a further 16 hours (control cells), or exposed to 10% FCS, 100 nM ET-1 or 100 μM PE for 16 hours. To determine protein synthesis rates, cells were labelled with [^{35}S]methionine/cysteine 1 hour before harvesting. To measure DNA synthesis rates, [^{3}H]thymidine was added to cells 16 hours before harvesting. Relative [^{35}S] and [^{3}H] incorporation was assessed by liquid scintillation counting. The fold increases represent the mean of five separate experiments, each with three replicates per condition. The error bars indicate the standard deviation from the mean (*significantly different from control, $p<0.02$; **not significantly different from control).

Figure 3.3 demonstrates that cardiomyocytes exposed to FCS for 16 hours were significantly larger (1.3-fold) than control, serum-starved cells. Consistent with this being part of a hypertrophic growth response, cell number was unaffected.

The mammalian heart contains a variety of cell types, in addition to cardiomyocytes, including fibroblasts and endothelial cells. Although the cardiomyocyte isolation procedure minimises the content of these cells in the final cultures, it is very difficult to completely exclude these cells. Unlike cardiomyocytes, cardiac fibroblasts can divide rapidly, and so if proliferation of these cells takes place, they may eventually overgrow the cardiomyocytes. Therefore, it is important to limit the proliferation of any contaminating fibroblasts within cardiomyocyte cultures. In this study, and many others, high plating density was used to restrict fibroblast proliferation (Sadoshima and Izumo, 1995; Sadoshima et al, 1997). Additionally, prior to use, cultures were maintained in media containing horse serum only, which is low in mitogens, or no serum. Figures 3.2 and 3.3 show that FCS does not induce detectable DNA synthesis or cell division in these cultures. Not only does this confirm that the isolated myocytes were non-dividing, but also suggests that the measures outlined above were sufficient in preventing the proliferation of any contaminating fibroblast cells.

In addition to changes in protein synthesis and cell size, a specific feature of the pathological hypertrophic growth response is the re-expression of characteristic foetal genes. In particular, induction of ANF expression has been demonstrated

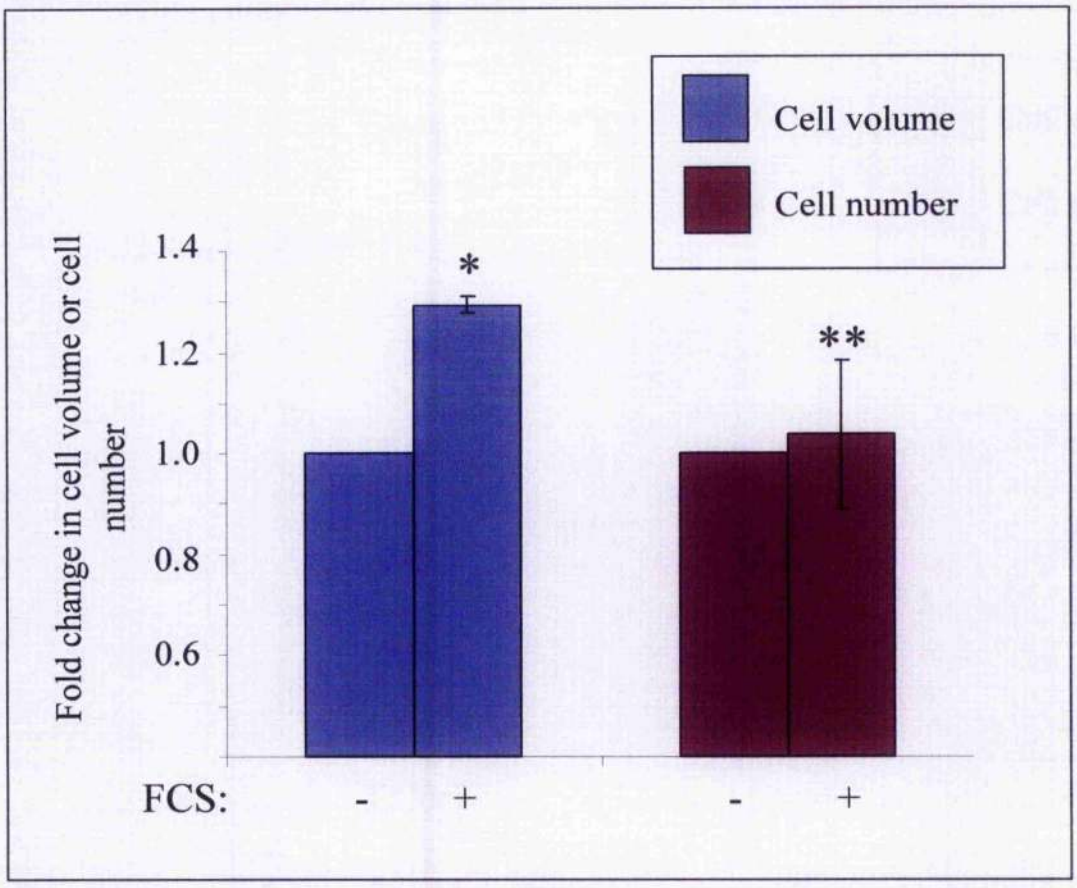


Figure 3.3: Stimulation of cultured cardiomyocytes with FCS increases cell size but not cell number. Cardiomyocytes were cultured in the absence of serum for 24 hours, then either maintained in serum-free media for a further 16 hours (control cells), or stimulated with 10% FCS for 16 hours. Cells were dissociated from dishes, then cell volume and number were measured using a Z2 Coulter counter. The fold increases represent the mean of three separate experiments, each with two replicates per condition. The error bars indicate the standard deviation from the mean (*significantly different from control, $p<0.05$; **not significantly different from control).

in a wide variety of species, in response to many stimuli (Chien et al, 1991; Hoshijima et al, 2004; Hunter and Chien, 1999). As a result, ANF expression is often used as a marker of hypertrophy in the heart and in primary cultured cell models. RT-PCR analysis confirmed that ANF expression was increased in response to FCS, ET-1 and PE treatment of the primary cultures used in this study (Figure 3.4). This effect was specific, as there was no change in the control mRNA, ARPP P0.

Thus, these cells display several defining features of the hypertrophic growth response, and so provide an appropriate culture system to determine whether pol III transcription is activated during cardiomyocyte hypertrophy.

3.2.2 Hypertrophic stimulation of cultured cardiomyocytes induces pol III transcription

Several techniques were used to assess changes in pol III transcription in response to hypertrophic stimulation of cultured cardiomyocytes. For example, Northern blotting demonstrated that induction of hypertrophic growth, with FCS, ET-1 or PE, is accompanied by a significant increase in the level of class III transcripts derived from the B2 middle repetitive gene family (Figure 3.5). This effect is specific, since the level of ARPP P0 mRNA is unaffected by these treatments. B2 transcripts have very short half-lives (less than 5 minutes (Bladon et al, 1990)), and therefore an increase in their abundance is indicative of an enhanced rate of ongoing transcription by pol III.

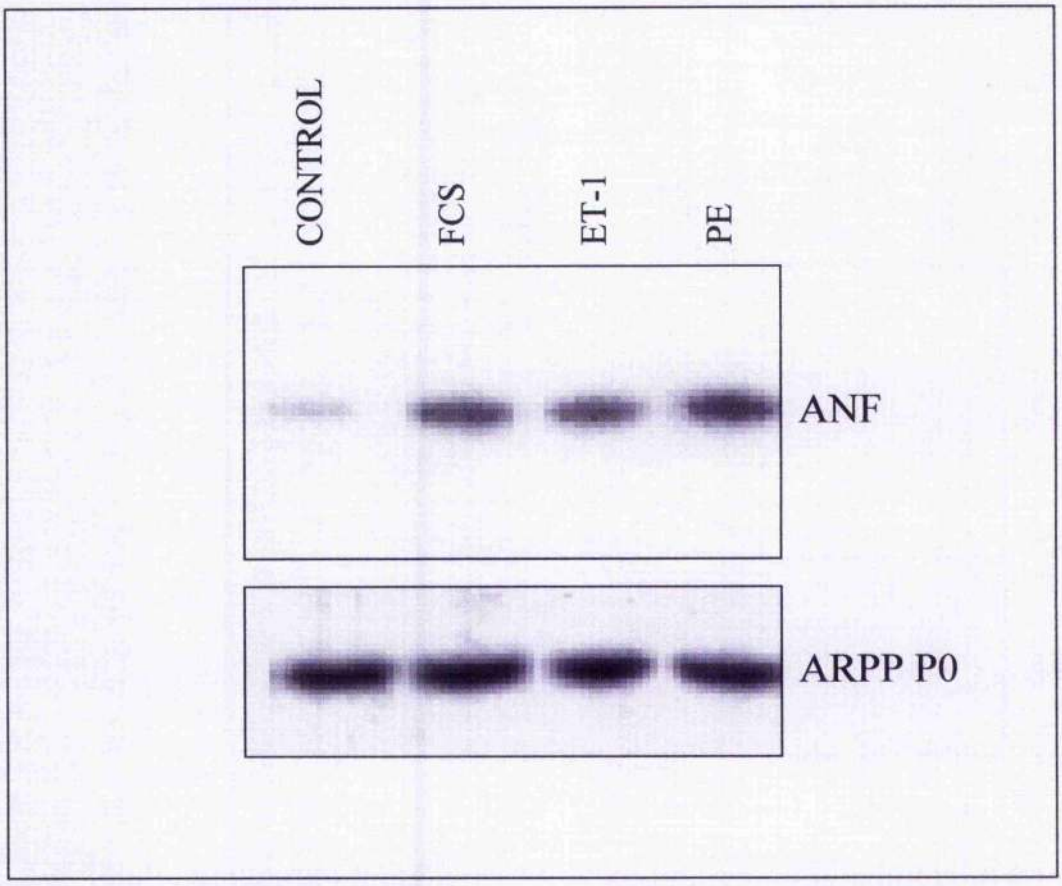
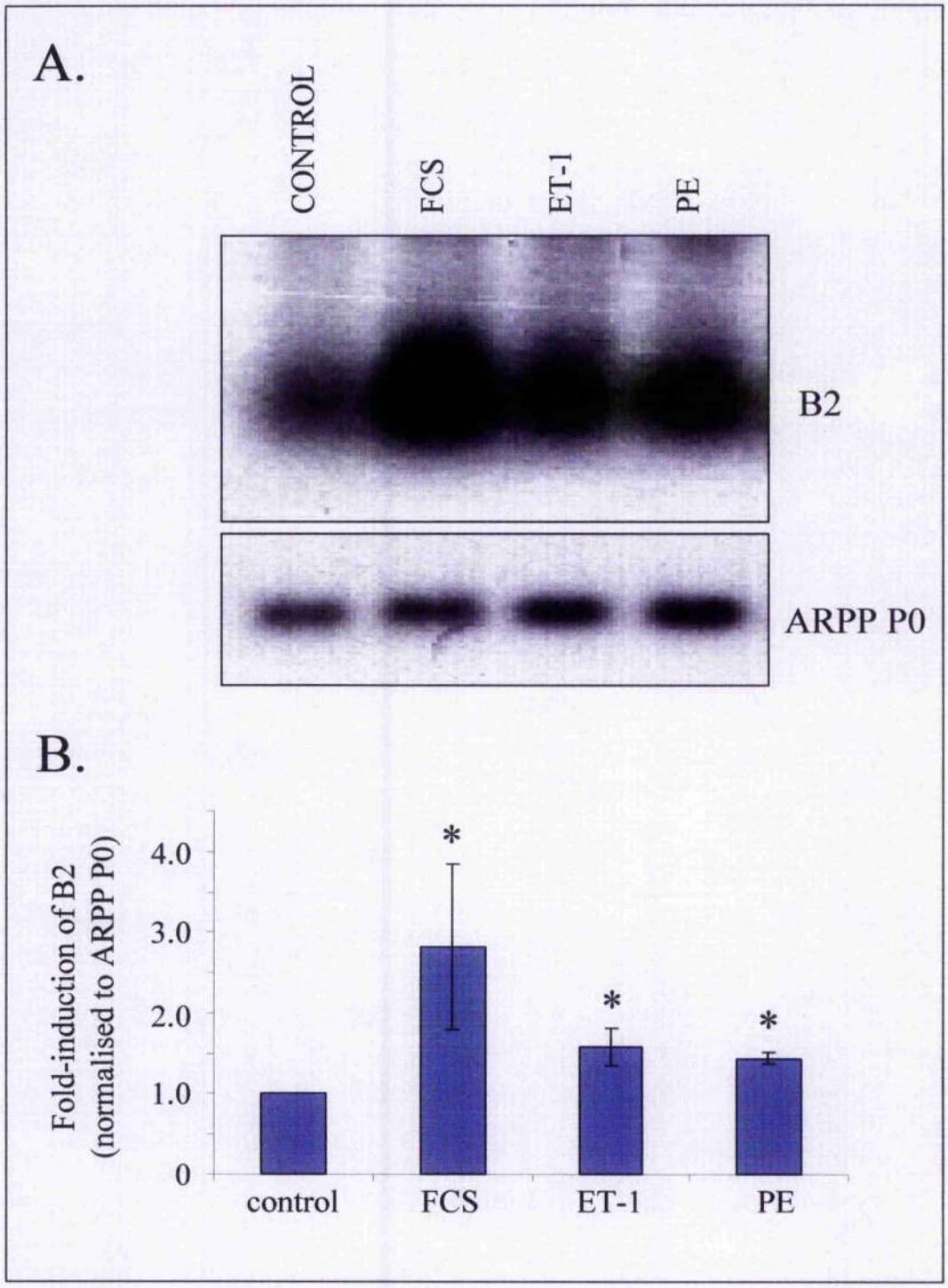


Figure 3.4: Various stimuli induce re-expression of the foetal gene ANF in cardiomyocyte cultures. Cultured cardiomyocytes were serum-starved for 24 hours, then either maintained in serum-free media for a further 16 hours (control cells), or exposed to 10% FCS, 100 nM ET-1 or 100 μM PE for 16 hours. Total RNA was extracted from these cells and 3 μg used to generate cDNAs by reverse transcription. Specific cDNAs were then amplified by PCR using ANF and ARPP P0 primers. The figure shows a representative RT-PCR analysis.

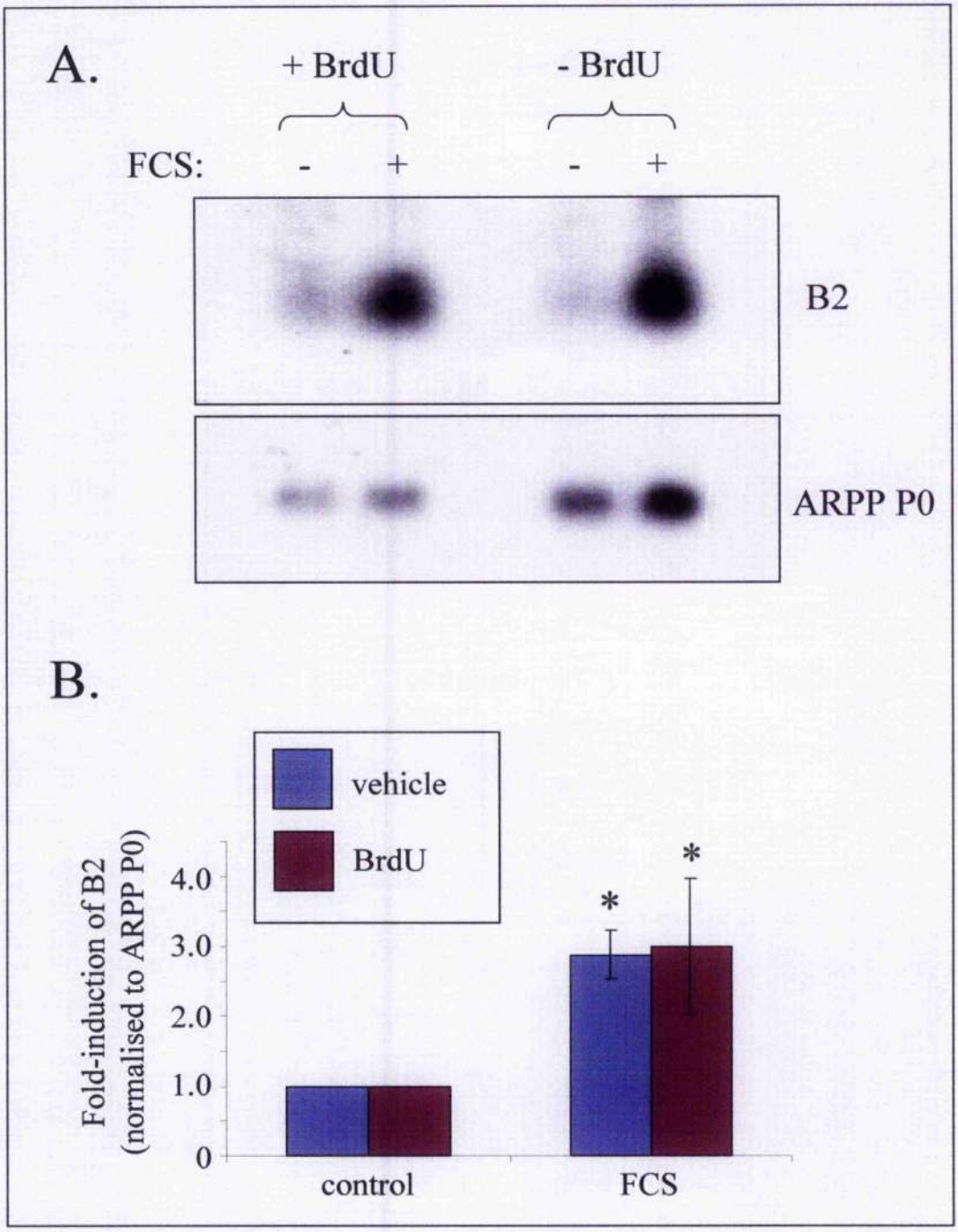
Figure 3.5: Hypertrophic stimulation of cultured cardiomyocytes induces B2 expression. Cultured cardiomyocytes were serum-starved for 24 hours. Hypertrophy was then induced by treating cells with 10% FCS, 100 nM ET-1 or 100 μM PE for 16 hours. Control cells were maintained in serum-free media for these 16 hours. Total cellular RNA was extracted and 10 μg analysed by Northern blotting. A. Representative Northern blot. The upper panel shows the blot probed for B2, and the lower panel shows the same blot stripped and re-probed for ARPP P0. B. The levels of B2 and ARPP P0 in each sample were quantified using densitometry, and B2 levels normalised against ARPP P0 levels. This was done for 3 separate experiments and the average fold increases obtained, along with standard deviations from the means, are represented in the graph (*significantly different from control, $p<0.05$).


A.
CONTROL
FCS
ET-1
PE
B2
ARPP P0
B.
Fold-induction of B2
(normalised to ARPP P0)
4.0
3.0
2.0
1.0
0
*
*
*
control
FCS
ET-1
PE

FCS has been shown to induce pol III transcription in proliferating fibroblast cells (Felton-Edkins et al, 2003a; Johnson et al, 1974; Mauck and Green, 1974; Scott et al, 2001). The lack of effect of FCS on DNA synthesis (Figure 3.2) and cell number (Figure 3.3) in this study indicated that there was no significant pool of proliferating fibroblasts within the cultures used, as discussed previously. Nonetheless, it was important to ascertain that the observed increase in class III transcripts, in response to FCS, was a feature of the hypertrophic growth of the myocyte population, and not associated with any contaminating fibroblast proliferation. In addition to high plating density and the use of mitogen-poor serum, some investigators also include BrdU in cardiomyocyte culture media (Bogoyevitch et al, 1993; Boluyt et al, 1997; McDermott et al, 1989; Sadoshima and Izumo, 1995; Simpson et al, 1982; Starsken et al, 1986). BrdU is toxic to dividing cells, and therefore ensures that fibroblast proliferation is inhibited (Simpson and Savion, 1982). However, BrdU does not influence the stimulation of cardiomyocyte hypertrophic growth (Simpson et al, 1982). Although BrdU was not routinely used in the current study, to rule out the possibility that the increase in pol III transcripts in response to FCS was due to contaminating fibroblast cells, the effects of FCS on B2 induction were compared in the presence and absence of BrdU (Figure 3.6). Inclusion of BrdU in the culture media had no significant effect on the induction of B2 by FCS, as determined by Northern blotting, thus confirming that proliferating cells do not contribute to this increase in class III transcripts.

As described in Chapter 1, pol III transcribes a variety of genes in addition to B2. To assess the effect of hypertrophic stimuli on the expression of a range of other

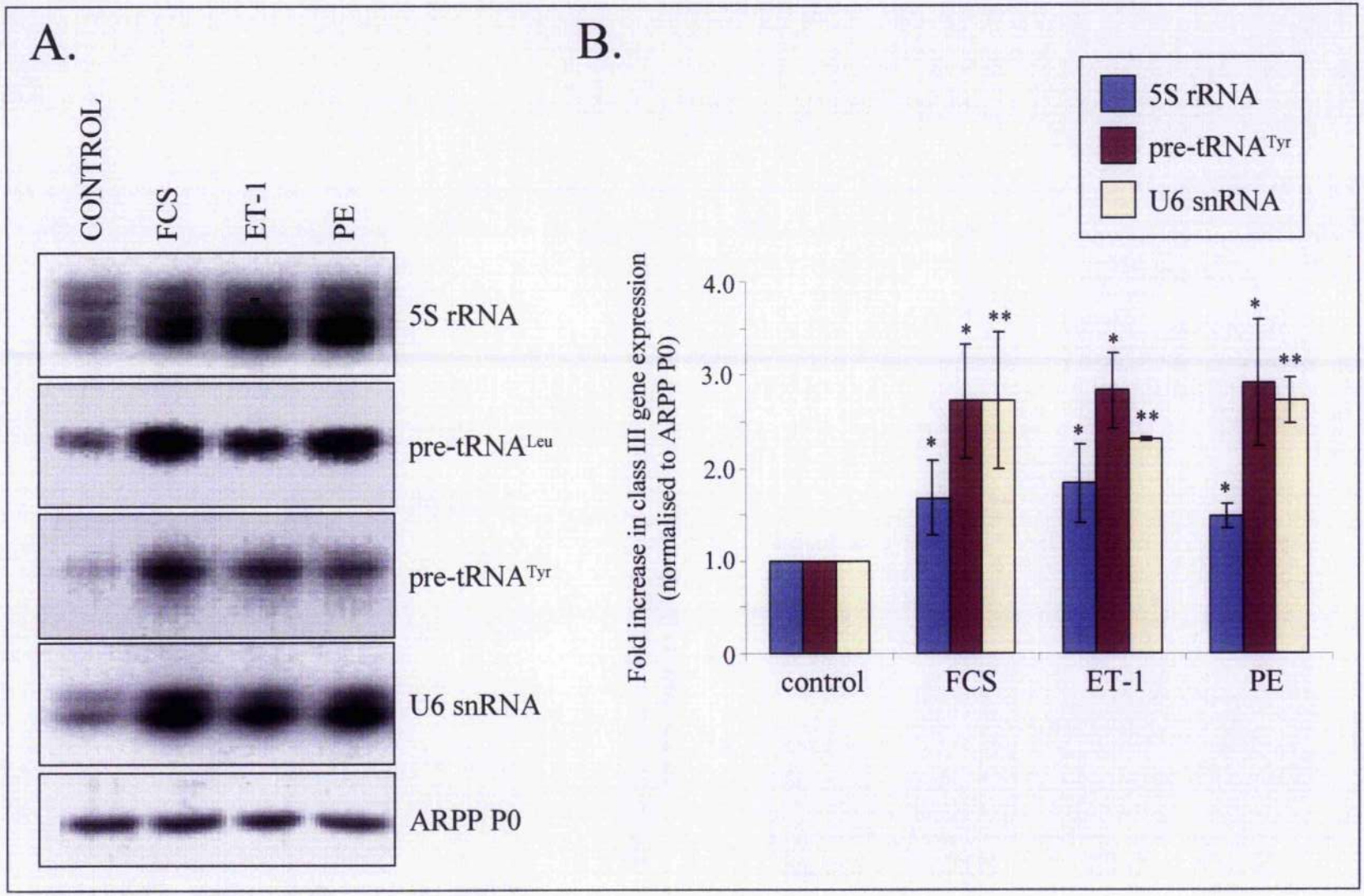
Figure 3.6: BrdU has no effect on B2 induction by FCS in cultured cardiomyocytes. Following dissociation from neonatal rat hearts, cardiomyocytes were cultured in media containing 0.1 mM BrdU or vehicle alone (0.1% DMSO). BrdU and vehicle exposed cells were serum starved for 24 hours, then treated with or without 10% FCS to induce hypertrophy. BrdU or vehicle were present throughout the course of the experiment. 10 μg of total RNA was analysed by Northern blotting. A. Representative Northern blot. The upper and lower panels show the blot probed for B2 then re-probed for ARPP P0, respectively. B. B2 and ARPP P0 levels were quantified using densitometry, and B2 normalised against ARPP P0. This was done for 4 experiments and the average fold increases obtained are represented in the graph. The error bars indicate the standard deviation from the mean (*both are significantly different from control, $p<0.05$, but not significantly different from each other).


A.
+ BrdU
- BrdU
FCS:
-
+
-
+
B2
ARPP P0
B.
vehicle
BrdU
Fold-induction of B2
(normalised to ARPP P0)
4.0
3.0
2.0
1.0
0
*
*
control
FCS

pol III-transcribed genes, semi-quantitative RT-PCR analysis was used. As before, hypertrophy was induced in cultured cardiomyocytes by exposing serum-starved cells to FCS, ET-1 or PE for 16 hours. Extracted RNA was then analysed by RT-PCR using primers specific for various pol III templates. This revealed an increase in all the pol III transcripts tested, in stimulated cells compared to control cells, including 5S rRNA, U6 snRNA and precursors for tRNAs tyrosine (pre-tRNA^{Tyr}) and leucine (pre-tRNA^{Leu}) (Figure 3.7). The primers used to measure tRNA levels were designed to hybridise to rapidly processed introns within the nascent tRNA transcripts (Winter et al, 2000). Therefore, whereas the 5S rRNA and U6 snRNA primers measure steady-state transcript levels, the levels of pre-tRNA^{Tyr} and pre-tRNA^{Leu} provide a direct measure of transcriptional output (Winter et al, 2000). The genes transcribed by pol III can be categorised depending on their promoter structure, of which there are three types (types 1-3). 5S rRNA, tRNA and U6 snRNA genes have type 1, 2 and 3 promoters, respectively. Therefore, this analysis suggests that there is a general increase in pol III transcription during hypertrophic growth, which is not specific to one particular pol III template or promoter type.

The increase in various pol III transcripts following hypertrophic stimulation, as determined by Northern blotting and RT-PCR analysis, indicated that pol III transcription was activated during hypertrophic growth. In particular, the increase in intron-containing tRNAs directly demonstrated an induction of transcription by pol III (Figure 3.7). This may have been due to an increase in the inherent activity of the pol III transcription machinery or, for example, as a result of altered chromatin structure and hence increased accessibility to these

Figure 3.7: Hypertrophic stimulation of cultured cardiomyocytes induces the expression of various class III genes. Cultured cardiomyocytes were treated as in Figure 3.5 to induce hypertrophy. 3 μg of total cellular RNA was used to generate cDNAs by reverse transcription. cDNAs were then PCR amplified using primers specific for 5S rRNA, pre-tRNA^{Leu}, pre-tRNA^{Tyr}, U6 snRNA and ARPP P0, as indicated. A. Representative RT-PCRs are shown. B. Transcript intensities were quantified and the level of class III gene products were normalised to ARPP P0. The average fold increases for each class III transcript are shown in the graph. The error bars indicate the standard deviation from the mean (*n=3 and significantly higher than control, $p<0.05$; **n=2).


A.
CONTROL
FCS
ET-1
PE
5S rRNA
pre-tRNA^Leu
pre-tRNA^Tyr
U6 snRNA
ARPP P0
B.
5S rRNA
pre-tRNA^Tyr
U6 snRNA
Fold increase in class III gene expression (normalised to ARPP P0)
4.0
3.0
2.0
1.0
0
control
FCS
ET-1
PE

templates. To directly test whether the pol III transcription machinery was more active following hypertrophic stimulation, IVTs were performed. Whole-cell extracts were prepared from FCS-starved and FCS-stimulated cells and tested for their ability to support transcription of various pol III templates *in vitro*, including 5S rRNA, B2, $tRNA^{Leu}$ and adenovirus VAI (Figure 3.8). This showed that pol III transcriptional activity was higher in extracts derived from hypertrophic cardiomyocytes, thus supporting the conclusion that pol III transcription is activated during cardiomyocyte hypertrophy in culture.

Increased transcription is often associated with augmented polymerase recruitment to target genes (White, 2001). To examine whether this was true for pol III in cardiomyocytes, ChIPs were performed using antibodies against the largest pol III subunit and, as negative controls, a TFIIB antibody or beads alone. Figure 3.9 shows that, although hypertrophic stimulation of cardiomyocytes had little effect on the level of input genomic DNA, there was a marked increase in pol III binding to 5S rRNA and tRNA genes *in vivo*. This is consistent with the hypertrophy-associated increase in transcription of these genes (Figures 3.7 and 3.8).

Thus, hypertrophic stimulation of cultured cardiomyocytes enhances pol III binding to promoters *in vivo*, activates the pol III transcription machinery and hence induces the expression of various class III genes.

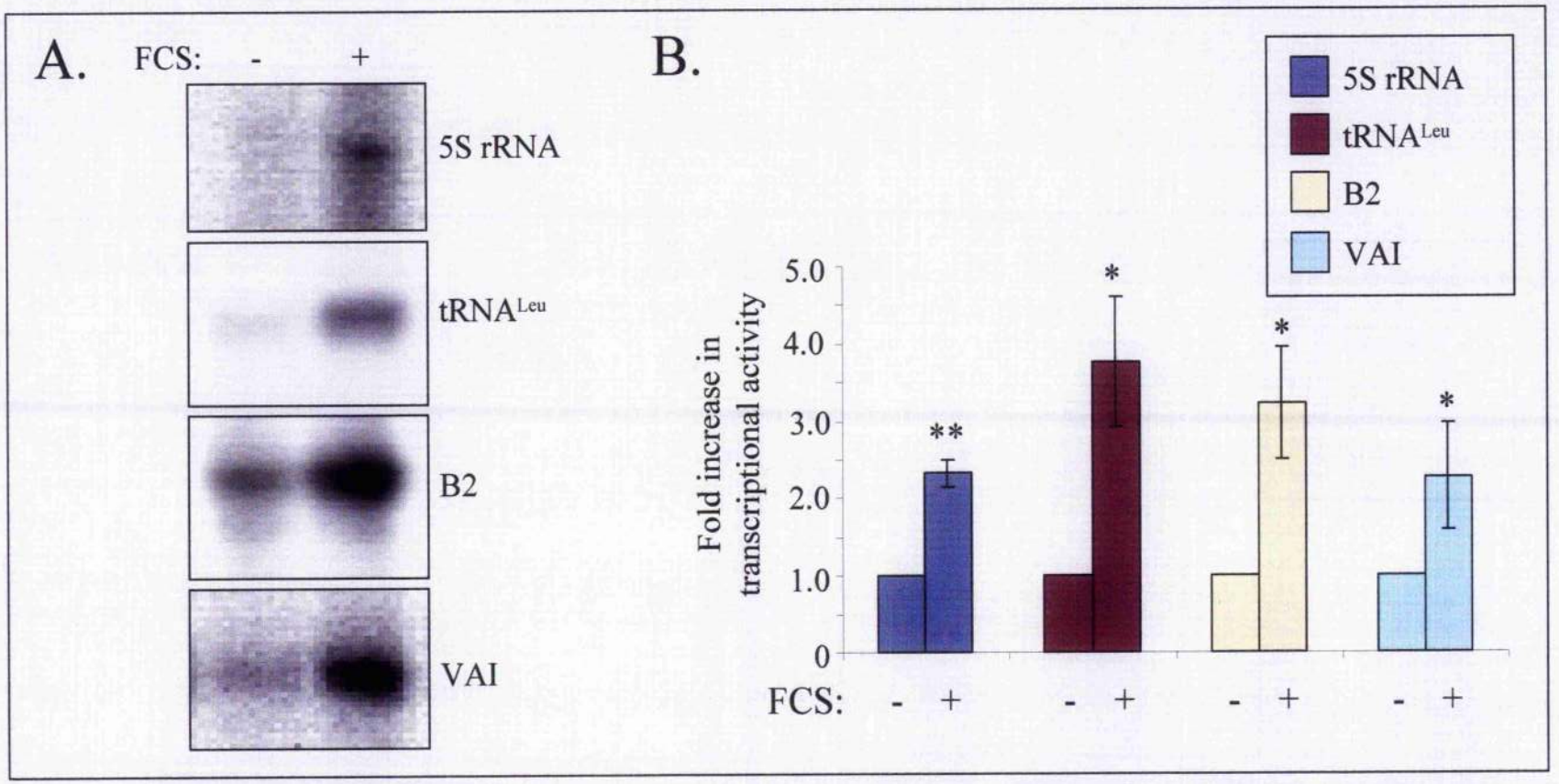


Figure 3.8: Hypertrophic stimulation of cultured cardiomyocytes increases the activity of the pol III transcription machinery. 20μg of whole cell extracts, prepared from serum-starved or FCS-stimulated cardiomyocytes, were tested for their ability to support transcription of 5S rRNA, $tRNA^{Leu}$, B2 and VAI templates *in vitro*. A. Representative IVTs are shown. B. The fold increases in transcriptional activity, towards each of these templates, were quantified and are displayed graphically. The error bars indicate the standard deviation from the mean (*n=3 and significantly higher than control, $p<0.05$; **n=2).

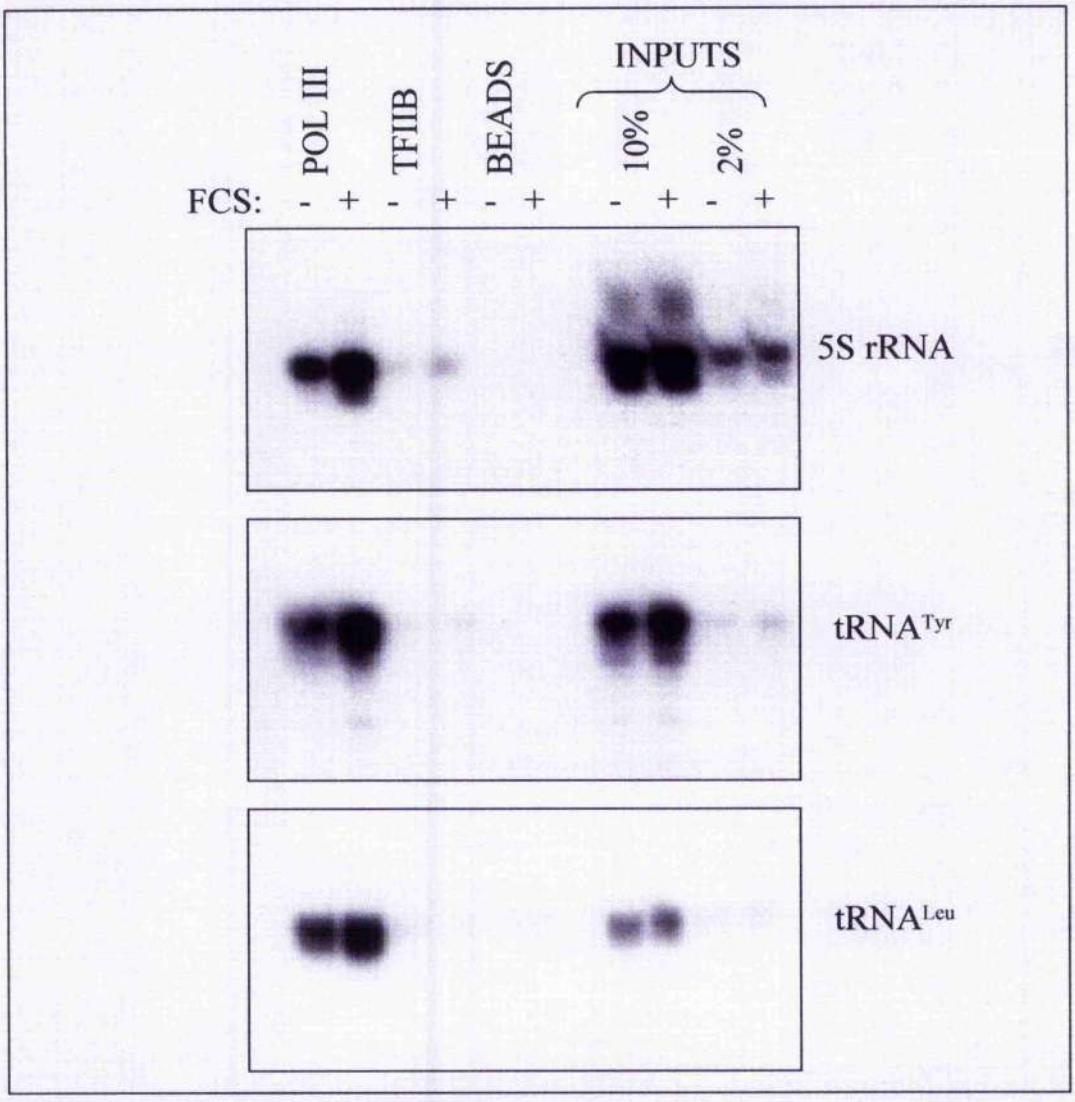
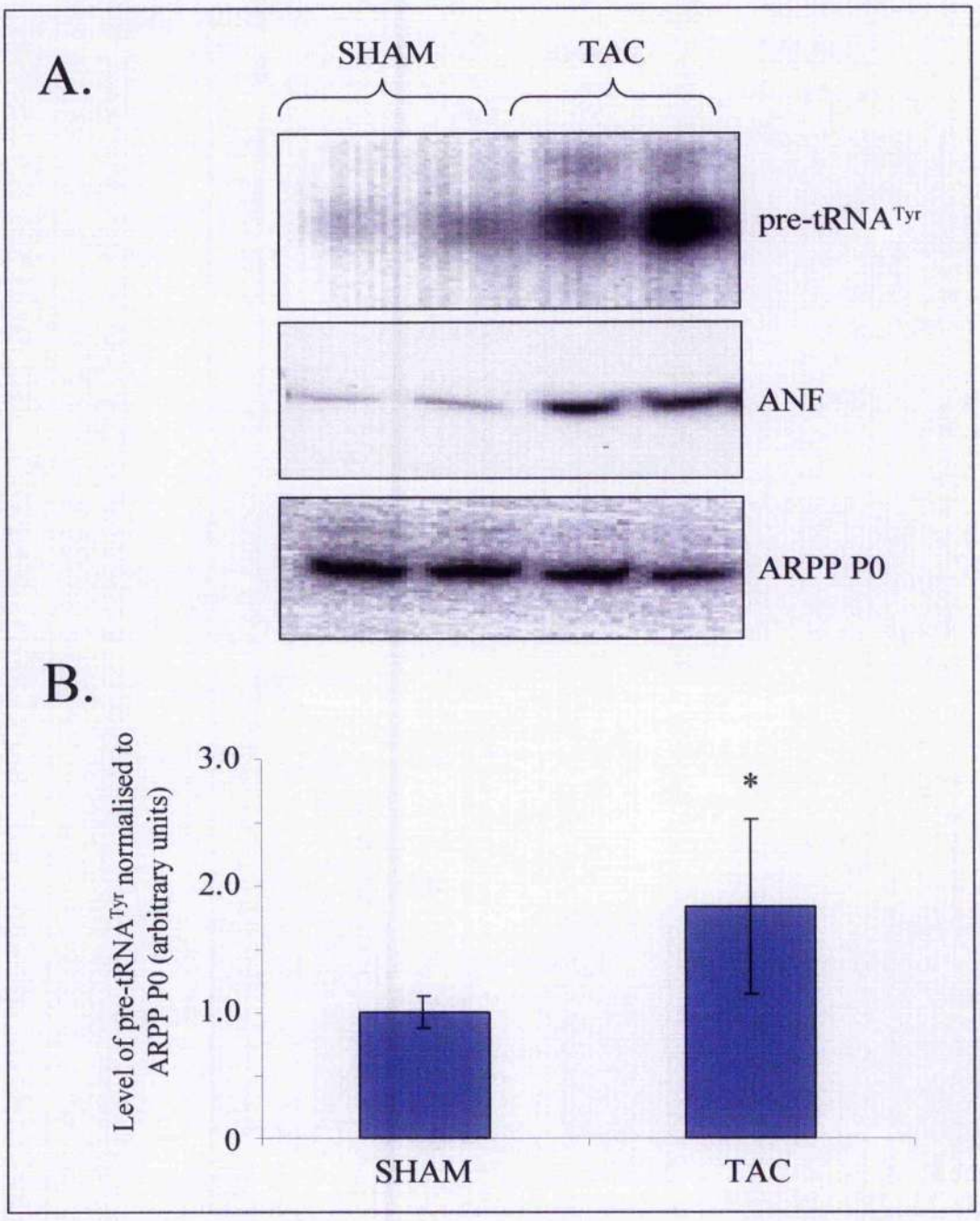


Figure 3.9: Hypertrophic stimulation of cultured cardiomyocytes enhances pol III binding to class III gene promoters *in vivo*. Hypertrophic growth was induced in cultured cardiomyocytes, as before, with 10% FCS. Chromatin was then isolated from control, serum-starved cells and FCS-stimulated cells. ChIPs were performed using Protein A Sepharose beads alone, as a control, or beads coupled to antibodies against TFIIB (irrelevant antibody control) or the largest pol III subunit, as indicated. Association of pol III with 5S rRNA, $tRNA^{Leu}$ and $tRNA^{Tyr}$ promoters in unstimulated and hypertrophic cells was quantified by PCR with gene-specific primers. Input genomic DNA (10% and 2% of that used in the ChIPs) was analysed in the same PCR reactions. The figure shown is representative of at least 3 independent ChIP experiments.

3.2.3 Hypertrophic stimulation of the heart induces pol III transcription in mice

In addition to cultured cells, animal models are also invaluable for studying the causes and consequences of myocardial growth. Hypertrophy can be induced in the hearts of these animals by administering certain hormones, or by mimicking cardiac pressure overload using surgical procedures (Antos et al, 2001; Busk et al, 2002; Nozato et al, 2001; Rockman et al, 1994; Shioi et al, 2003). For example, pressure overload hypertrophy is commonly induced in mice by chronic constriction of the transverse aorta (Rockman et al, 1991). This causes an increase in cardiomyocyte size and hence myocardial mass, along with characteristic changes in gene expression (Rockman et al, 1991). To assess whether pol III transcription was activated during this hypertrophic response, hearts were obtained from adult mice in which pressure overload hypertrophy had been induced by transverse aortic constriction (TAC) for 2 weeks (in the laboratory of Dr WR MacLellan, UCLA, USA). Control mice were subjected to sham operation. RNA was extracted from these hearts and analysed by RT-PCR. TAC caused a clear increase in ANF mRNA levels, confirming that this procedure induced a hypertrophic response (Figure 3.10). This was accompanied by a significant increase in the level of pre-tRNA^{Tyr} transcripts (Figure 3.10), thus demonstrating that pol III transcription is not only activated during cardiomyocyte hypertrophy in culture, but also in the heart.

Figure 3.10: Hypertrophic stimulation of the heart activates pol III transcription in mice. Hearts were obtained from mice in which pressure-overload hypertrophy had been induced by TAC for 2 weeks. Control animals underwent sham operation (SHAM). RNA was extracted from whole hearts, DNase I-treated, then used for RT-PCR analysis with pre-tRNA^{Tyr}, ANF and ARPP P0 specific primers. A. RT-PCRs, using RNA derived from 2 separate animals per condition, are shown (SHAM (lanes 1 and 2) or TAC (lanes 3 and 4)). B. Densitometry was used to quantify the amounts of pre-tRNA^{Tyr} and ARPP P0 in each heart. Six hearts were analysed for each condition, and the average level of pre-tRNA^{Tyr}, normalised to ARPP P0, is shown in the graph. The error bars indicate the standard deviation from the mean (*significantly higher than control, $p<0.05$). The hearts used for these analyses were provided by Dr WR MacLellan, University of California, Los Angeles, USA.


A.
SHAM
TAC
pre-tRNA^{Tyr}
ANF
ARPP P0
B.
Level of pre-tRNA^{Tyr} normalised to ARPP P0 (arbitrary units)
3.0
2.0
1.0
0
*
SHAM
TAC

3.3 Discussion

Heart growth in adults, in response to increased demands, occurs through cardiomyocyte hypertrophy. Accelerated rates of protein synthesis, leading to protein accumulation, facilitate this cell growth (Morgan et al, 1987). However, the mechanisms by which hypertrophic stimuli cause this increased synthesis are incompletely defined. Pol III produces essential components of the cellular biosynthetic machinery, such as 5S rRNA and tRNAs. Thus, by targeting the pol III transcriptional apparatus, hypertrophic stimuli could potentially increase the protein synthetic capacity of cardiomyocytes, making the pol III transcription machinery a likely candidate for regulation during hypertrophic growth. The data presented in this chapter confirmed that hypertrophic growth, characterised by increased protein synthesis and cell size in the absence of cell division, and the re-expression of the foetal gene, ANF, can be induced in primary cultured cardiomyocytes. Northern blotting, RT-PCR, IVTs and ChIP analysis revealed that this hypertrophic growth was accompanied by an activation of the pol III transcription machinery, enhanced binding of pol III to promoters *in vivo*, and a general increase in class III gene expression. Analysis of hearts from mice, in which TAC had induced pressure-overload hypertrophy, indicated that this activation of pol III transcription was physiologically relevant. Thus, this provides the first, comprehensive analysis of changes in pol III transcription occurring during hypertrophic growth, and may represent a novel mechanism contributing to the increased rates of protein synthesis necessary to sustain cardiomyocyte hypertrophy (Figure 3.11). Not only does this add to the current understanding of a process with relevance to disease, but also indicates that

activation of pol III transcription can be associated purely with cell growth, without cell division.

An early study by Cutilletta et al (1978) demonstrated that aortic constriction increased the activity of all three RNA polymerases in cardiomyocytes. These investigators exposed isolated nuclei to varying concentrations of α–amanitin (derived from the fungus *Amanita phalloides*) in order to distinguish between the activities of pols I, II and III, which have differential sensitivities to this toxin. This assay was relatively crude and, furthermore, the consequences of increased enzymatic activity on changes in class I, II or III gene expression were not addressed. A more recent investigation reported increased transcription of the MRP gene by pol III under hypertrophic conditions in cultured cardiomyocytes (Hannan and Rothblum, 1995). MRP is involved in processing the pre-rRNAs produced by pol I. However, the relevance of this observation to hypertrophic growth, or whether it was due to a general increase in pol III transcription, was not explored. The current study extends these limited observations described above, and presents a more thorough examination of hypertrophy-associated class III gene expression. Crucially, increased expression of 5S rRNA and tRNA was clearly demonstrated here, suggesting that the increase in pol III transcription is likely to have functional consequences for protein synthetic capacity.

Various factors influence the extent of hypertrophic growth, including the nature and duration of the initiating stimulus. The former is exemplified in Figure 3.2: FCS increased protein synthesis by approximately 2.4-fold, whereas ET-1 and

PE caused only 1.7- and 1.65-fold increases, respectively. This is consistent with previous studies, which have also demonstrated that FCS is a more potent inducer of hypertrophy than various individual agonists (Busk et al, 2002; Simpson et al 1982; Taigen et al, 2000). This may be due to the presence of several, synergistically acting hormones and growth factors in FCS (Simpson et al, 1982). Consequently, for all subsequent analyses of the mechanisms regulating pol III transcription in cultured cardiomyocytes, FCS was used as the hypertrophic stimulus.

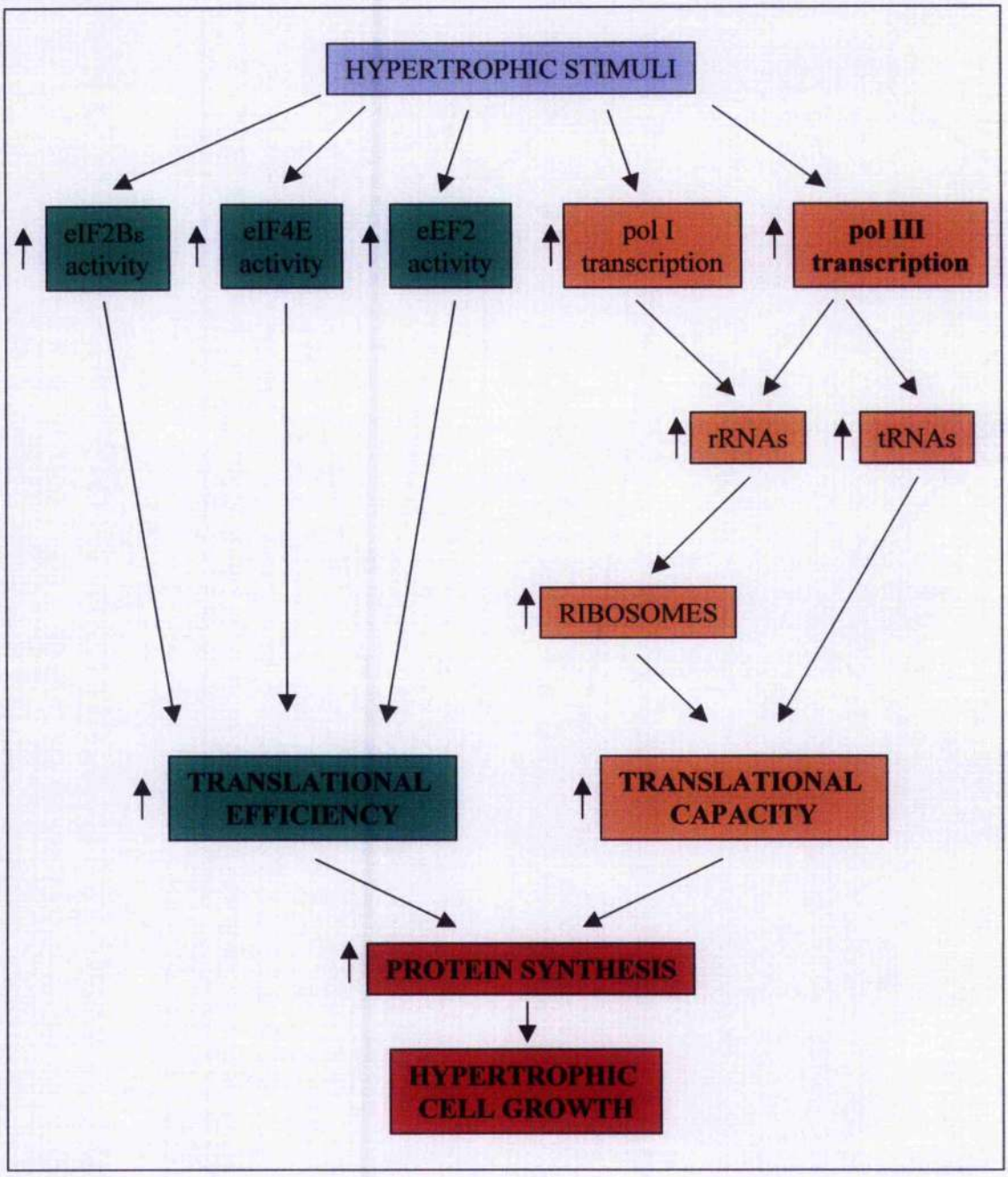
Stimulation of cardiomyocytes in culture with 10% FCS for 16 hours caused a significant, 1.3-fold increase in cell volume in the current study (Figure 3.3). Hypertrophic stimulation of cultured cardiomyocytes with a variety of agents, for various lengths of time, has been reported to induce increases in cell surface area ranging from approximately 1.2- to 2.3-fold (Boluyt, et al, 1997; Brandenburger et al, 2001; Busk et al, 2002; Glennon et al, 1996; Haq et al, 2003; Nozato et al, 2001; Taigen et al, 2000; Tamamori et al, 1998; Yanazume et al, 2003). Although direct comparisons of these values are not possible, as different conditions for inducing hypertrophy and techniques for measuring cell size were used, the fold-increase reported here does fall within this range. In studies where cell enlargement exceeded that observed here, cells had been exposed to hypertrophic stimuli for a minimum of 48 hours.

It is generally accepted that cardiomyocytes irreversibly withdraw from the cell cycle shortly after birth in mammals, and become terminally differentiated. However, recent studies have challenged this view, and have presented evidence

to suggest that cardiomyocyte proliferation can occur in the adult heart (Anversa and Kajstura, 1998; Anversa and Nadal-Grinard, 2002; Beltrami et al, 2001). This issue remains controversial and, in the main, cardiomyocyte hyperplasia is considered to be a rare event, if occurring at all. For the purposes of this study, cardiomyocytes were cultured from neonatal rat hearts. Although it is possible that, under some conditions, these cells may show limited capacity to proliferate, analysis of changes in [^{3}H]thymidine incorporation (Figure 3.2) and cell number (Figure 3.3) in response to serum stimulation, confirmed that such cell division was not present in the primary cells used here. Likewise, no evidence of myocyte cell cycle re-entry was detected in hearts following TAC (MacLellan, unpublished data). Therefore, the systems used were *bona fide* models for studying hypertrophic growth without cell division, thus validating the conclusion that pol III transcriptional activation was independent of proliferation.

The ways in which cardiomyocytes achieve increased rates of protein synthesis during hypertrophic growth are beginning to be elucidated, as summarised in Figure 3.11. In addition to reviewing the mechanisms previously implicated in mediating hypertrophic cell growth, this figure also illustrates the role pol III may play in this process. As previously discussed, it is an increase in translational capacity that is thought to be critical for sustained hypertrophic growth (Brandenburger et al, 2001; Morgan et al, 1987; Siehl et al, 1985). Along with pol I, enhanced pol III transcription will likely contribute to this increased capacity. Activation of transcription by pols I and III often coincide, in order to coordinate the production of ribosomes, tRNAs and other RNAs involved in cellular biosynthesis. This study suggests that the hypertrophic growth of

Figure 3.11: Mechanisms likely to contribute to increased rates of protein synthesis during cardiomyocyte hypertrophy. Hypertrophic stimulation of cardiomyocytes leads to the activation of the translation initiation factors, eIF4E and eIF2Bε, and the translation elongation factor, eEF2. This is likely to increase the efficiency with which existing ribosomes synthesise novel peptide chains. Other changes that occur following hypertrophic stimulation include increased pol I transcription, which is known to be essential for hypertrophic growth, and, as demonstrated in the current study, increased pol III transcription. The latter is also likely to contribute to an increase in translational capacity, and hence increased protein synthesis.


HYPERTROPHIC STIMULI
eIF2Bε activity
eIF4E activity
eEF2 activity
pol I transcription
pol III transcription
rRNAs
tRNAs
RIBOSOMES
TRANSLATIONAL EFFICIENCY
TRANSLATIONAL CAPACITY
PROTEIN SYNTHESIS
HYPERTROPHIC CELL GROWTH

cardiomyocytes provides no exception to this frequent co-regulation, and highlights the fundamental importance of such synchrony.

Having established that pol III transcription is activated during cardiomyocyte hypertrophy, the next aims of the project were to determine the mechanisms responsible for this increase. The following chapters examine whether the pol III transcription machinery is a target for various known mediators of hypertrophy, that have been shown to activate pol III transcription in immortalised, proliferating cell types. Novel mechanisms for regulating pol III transcription in cardiomyocytes are also considered.

CHAPTER 4
Several mechanisms potentially regulate TFIIIB activity during cardiomyocyte hypertrophy

4.1 Introduction

The data presented in Chapter 3 demonstrate that pol III transcription is activated during the hypertrophic growth of cardiomyocytes. The mechanisms controlling this increase are investigated in the remainder of this thesis. This chapter considers a variety of potential regulatory mechanisms, and aims to establish whether any of these could be involved in the control of pol III transcription during cardiomyocyte hypertrophy. An understanding of these mechanisms might be useful when devising strategies to manipulate hypertrophic growth in disease. In addition, because the majority of studies on the regulation of pol III transcription in mammalian cells have focused on dividing, immortalised cell types, it will be interesting to determine whether the same or distinct mechanisms operate in non-dividing, primary cells.

4.1.1 Regulation of pol III transcription

The transcription of a class III gene can be divided into three main stages: initiation, elongation and termination. The rate of transcription is primarily regulated at the level of initiation. This is dependent on the productive assembly of a transcription initiation complex at the appropriate promoter, and the subsequent recruitment of pol III. For the majority of class III genes, this process requires two pol III-specific, basal transcription factors: the promoter-recognition factor, TFIIIC, and the polymerase recruitment factor, TFIIIB (see Chapter 1;

Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). Several components of these transcription factor complexes are regulated under various circumstances in eukaryotic cells, in order to modulate pol III transcriptional output, as detailed in Chapter 1. In particular, TFIIIB activity is stringently controlled by a variety of mechanisms.

In proliferating cells, control of TFIIIB activity is predominantly achieved through protein-protein interactions with various regulators. For example, the MAP kinase ERK can induce pol III transcription by binding and phosphorylating the Brf1 subunit of TFIIIB (Felton-Edkins et al, 2003a). This enhances the interaction of TFIIIB with both TFIIIC and pol III, hence promoting pol III transcription (Felton-Edkins et al, 2003a). The proto-oncogene product c-Myc also activates pol III transcription, again by targeting TFIIIB (Gomez-Roman et al, 2003). In contrast to these positive regulators of pol III transcription, RB plays an important role in restraining pol III activity (White et al, 1996). In resting cells, RB binds and inactivates TFIIIB (Chu et al, 1997; Larminie et al, 1997; Sutcliffe et al, 2000), thereby preventing the unnecessary production of tRNAs, 5S rRNA and other pol III products. However, in response to mitogenic stimulation, RB becomes inactivated by CDK-induced hyperphosphorylation. This leads to the release of TFIIIB, and the activation of pol III transcription prior to S phase (Scott et al, 2001).

ERK, c-Myc and RB all play central roles in regulating mammalian cellular proliferation (for reviews see Chang et al, 2003; Dang, 1999; Kaelin, 1999; Lüscher, 2001; Weinberg, 1995; Wilkinson and Millar, 2000). However, it is

becoming increasingly apparent that these molecules are also important regulators of cell growth (Schmidt, 1999; White, 1998; Whitmarsh and Davis, 2000; Wilkinson and Millar, 2000), and in fact, under certain circumstances, this regulation can occur independently of cell division (Bueno et al, 2000; Glennon et al, 1996; Iritani and Eisenman, 1999; Johnston et al, 1999; Kim et al, 2000; MacLellan et al, unpublished results; Schuhmacher et al, 1999). This point is suitably illustrated by the fact that each of these proteins has been implicated in mediating the hypertrophic growth of cardiomyocytes (Bueno and Molkentin, 2002; MacLellan et al, unpublished results; Nozato et al, 2001; Sadoshima et al, 1997; Simpson, 1988; Tamamori et al, 1998; Xiao et al, 2001). The roles that these proteins play in the hypertrophic response are incompletely defined, but it seems highly possible that they will target the pol III transcription machinery in cardiomyocytes, and in this way regulate hypertrophic growth. Thus, ERK, c-Myc and RB are likely candidates for the regulation of pol III transcription during hypertrophy. To begin to address this possibility, this chapter examined the induction and/or phosphorylation of each of these potential regulators in cultured cardiomyocytes in response to hypertrophic stimulation.

In addition to changes in pol III transcription factor activity, alterations in transcription factor expression may also contribute to the regulation of pol III productivity. Changes in transcription factor abundance seem to be an important determinant of pol III transcription rates during certain instances of mammalian cell differentiation (Alzuherri and White, 1998), viral infection (Felton-Edkins and White, 2002; Larminie et al, 1999; Wang et al, 1995) and tumourigenesis (Winter et al, 2000; Daly et al, 2004, *In press*). Hypertrophy-associated changes

in the levels of pol III-specific transcription factors will be explored in this chapter.

In summary, the objectives of this chapter were to examine changes in the pol III transcription machinery, and proteins known to interact with this machinery, in response to hypertrophic stimulation, in order to establish a potential basis for the regulation of pol III transcription during cardiomyocyte hypertrophy.

4.2 Results

4.2.1 Interaction of the pol III transcription machinery with promoters *in vivo* during hypertrophic growth

Enhanced pol III transcription during hypertrophic growth accompanies an increase in polymerase binding to class III gene promoters *in vivo* (Figure 3.9), and this is likely to at least partly facilitate the observed activation of transcription by pol III. To investigate the basis of this increased pol III recruitment, ChIP assays were performed to examine the association of other components of the pol III transcription machinery with promoters during hypertrophy. As previously discussed, the recruitment of pol III to most target genes requires the transcription factor complexes TFIIIB and TFIIIC (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). To determine whether the binding of either of these factors to promoters was subject to regulation during hypertrophic growth, ChIP analyses were carried out using antibodies against two of the TFIIIB subunits (Brf1 and Bdp1), and two of the TFIIIC subunits (TFIIIC110 and TFIIIIC220). As shown in Figure 4.1, this demonstrated that, along with the previously described increase in pol III binding (Figure 3.9), hypertrophic stimulation (with FCS for 16 hours) also increases the level of TFIIIB bound to tRNA and 5S rRNA genes *in vivo*. Although in the example shown for $tRNA^{Tyr}$ the level of input DNA from serum-stimulated cells is also slightly higher, this difference is clearly less than the difference in TFIIIB immunoprecipitated under each condition. Increased TFIIIB binding is more striking for the tRNA genes than the 5S rRNA genes, and may reflect differences in the promoter types utilised by pol III to transcribe different templates (see Chapter 1). TFIIIC binding to each promoter type is unaffected. Figure 4.2

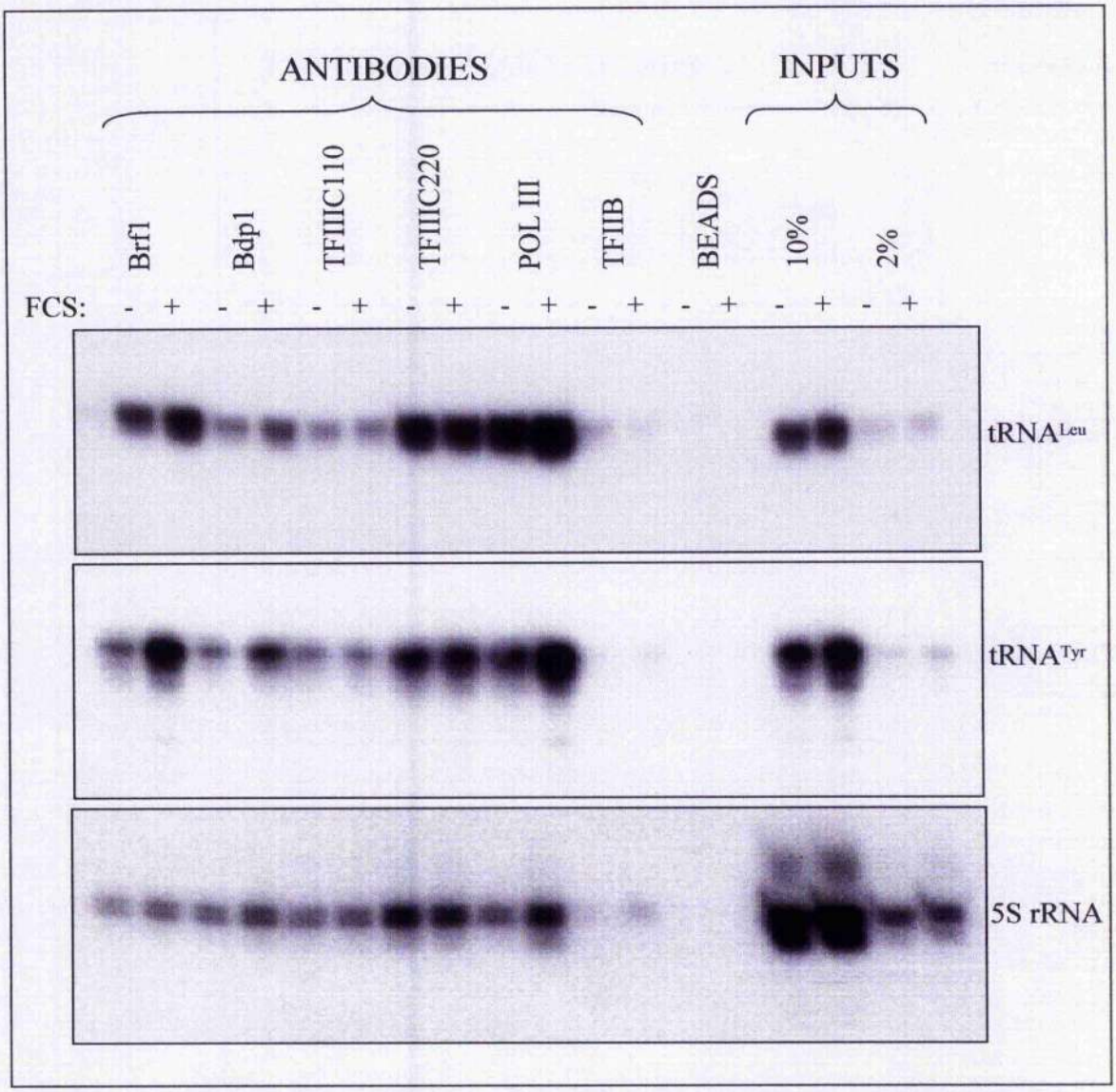


Figure 4.1: Hypertrophic stimulation of cardiomyocytes enhances TFIIIB and pol III, but not TFIIIC, binding to class III gene promoters *in vivo*. Cultured cardiomyocytes were treated with 10% FCS for 16 hours to induce hypertrophic growth. Control cells were maintained in serum-free medium. ChIPs were performed with antibodies against Brf1, Bdp1, TFIIIC110, TFIIIC220 and the largest pol III subunit, as indicated. As negative controls, ChIPs were carried out using a TFIIB antibody and beads alone. Association of each factor with 5S rRNA, $tRNA^{Leu}$ and $tRNA^{Tyr}$ promoters, in unstimulated and hypertrophic cells, was quantified by PCR with gene-specific primers. Input genomic DNA (10% and 2% of that used in the ChIPs) was analysed in the same PCR reactions. The figure shown is representative of 3 independent ChIP experiments.

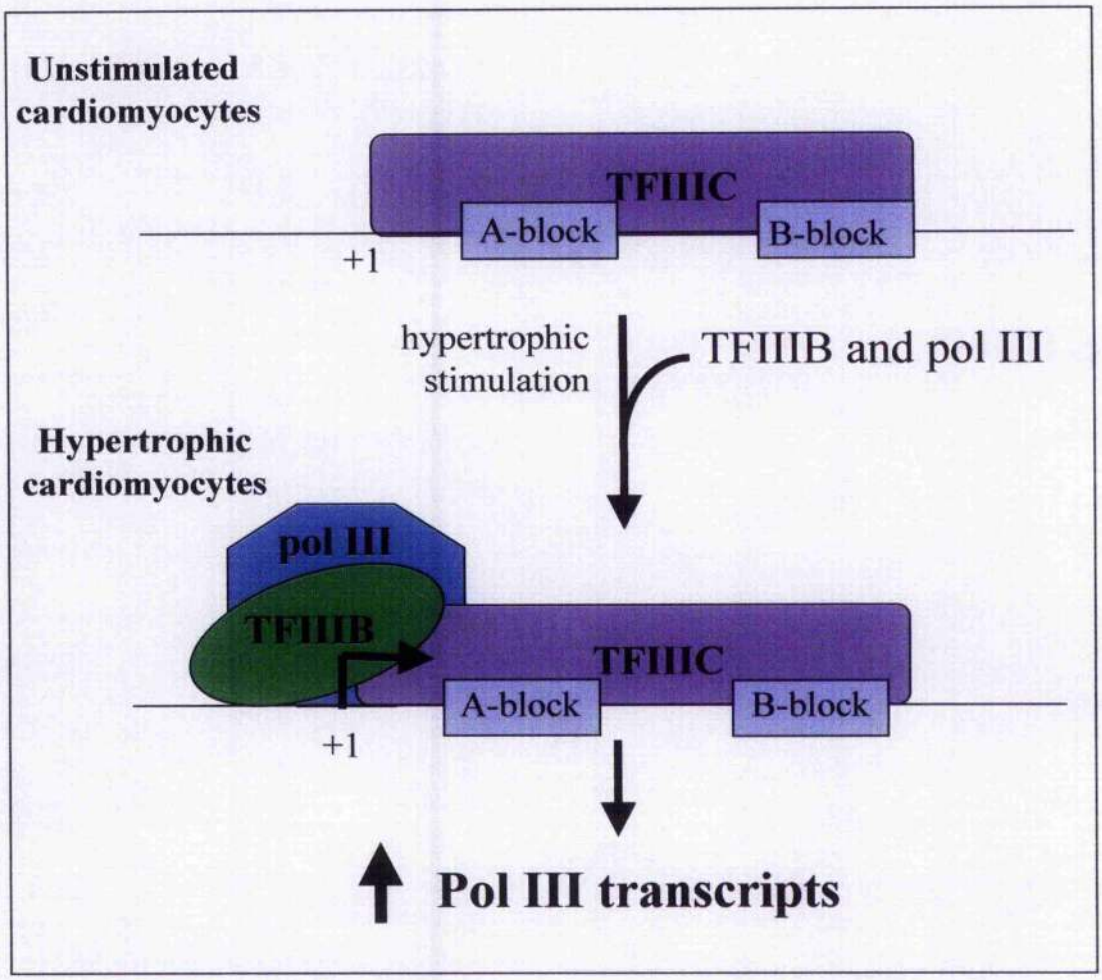


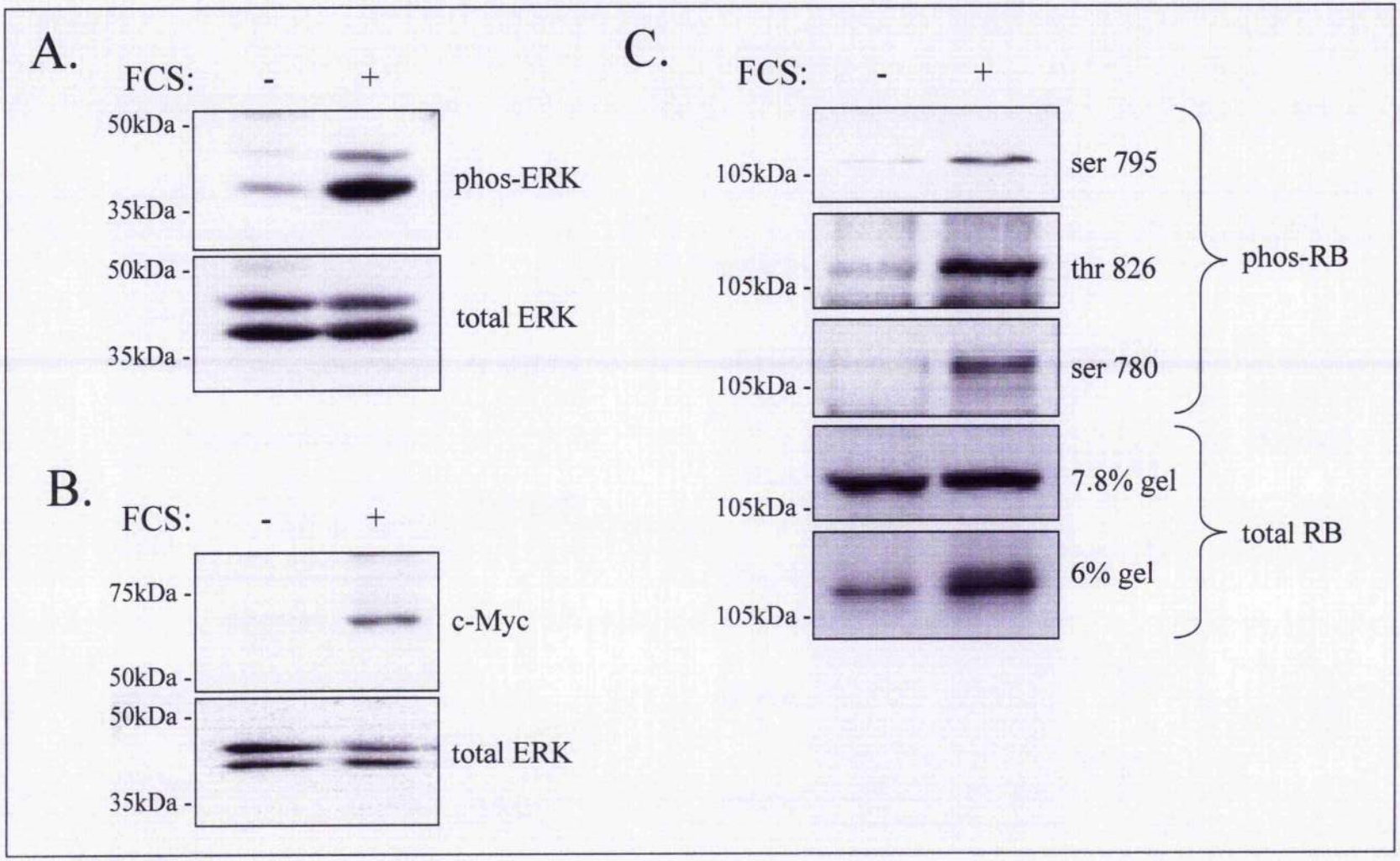
Figure 4.2: Increased TFIIIB and pol III recruitment to class III gene promoters may contribute to the activation of pol III transcription during cardiomyocyte hypertrophy. Hypertrophic stimulation of cardiomyocytes enhances the binding of TFIIIB to class III gene promoters *in vivo*. This is likely to facilitate the increase in pol III recruitment, and hence the activation of pol III transcription, that occurs during hypertrophic growth. A typical type 2 promoter is shown in this figure to illustrate these changes in promoter occupancy.

summarises these changes in class III gene promoter occupancy. TFIIIB is directly responsible for polymerase recruitment (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002); therefore, the increased association of TFIIIB with promoters is likely to account for the augmented polymerase binding, and hence the enhanced class III gene expression, that occurs during hypertrophic growth. This is consistent with TFIIIB being an important regulatory target for the control of pol III transcription in terminally differentiated cardiomyocytes.

4.2.2 Hypertrophic stimulation of cardiomyocytes influences known regulators of TFIIIB activity

As discussed above, TFIIIB activity may be a central determinant of the rate of pol III transcription during cardiomyocyte hypertrophy. Known regulators of pol III transcription in proliferating cells, which interact with TFIIIB, include ERK, c-Myc and RB (Chu et al, 1997; Felton-Edkins et al, 2003a; Gomez-Roman et al, 2003; Larminie et al, 1997; Sutcliffe et al, 2000). FCS was shown to induce pol III transcription and hypertrophic growth in primary cardiomyocyte cultures (Chapter 3). To establish whether ERK, c-Myc or RB could potentially contribute to this regulation of pol III transcription, protein lysates were prepared from cultured cardiomyocytes before and after hypertrophic stimulation with FCS for 16 hours. Western blotting was performed to assess the activation of ERK, the induction of c-Myc, and the phosphorylation status of RB (Figure 4.3).

Figure 4.3: Hypertrophic stimulation of cardiomyocytes influences known regulators of TFIIIB. Whole cell lysates were prepared from cultured cardiomyocytes that had been serum-starved for 24 hours, then either maintained in serum-free medium or exposed to 10% FCS for 16 hours. 75 μg of protein were then resolved by SDS-PAGE. Western blotting was performed with antibodies against the proteins, or phosphoproteins (indicated by 'phos-'), specified. A. Hypertrophic stimulation induces ERK phosphorylation. B. Hypertrophic stimulation induces c-Myc expression. The anti-c-Myc antibody 9E10 was used here. C. Hypertrophic stimulation induces the hyperphosphorylation of RB. The Western blots in the top three panels were probed with antibodies that specifically detect RB phosphorylated on serine (ser) 795, threonine (thr) 826, or ser 780. Total RB levels were measured after electrophoresis on both 7.8% (with antibody C-15) and 6% (with antibody G3-245) polyacrylamide gels (bottom two panels).


A.
FCS: - +
50kDa -
35kDa -
phos-ERK
50kDa -
35kDa -
total ERK
B.
FCS: - +
75kDa -
50kDa -
c-Myc
50kDa -
35kDa -
total ERK
C.
FCS: - +
105kDa -
ser 795
105kDa -
thr 826
105kDa -
ser 780
phos-RB
105kDa -
7.8% gel
105kDa -
6% gel
total RB

ERK activation results from the phosphorylation of two particular residues by upstream kinases (Garrington and Johnson, 1999). Using a phospho-specific antibody, that recognises the 44 and 42kDa isoforms of activated ERK, Western blotting demonstrated ERK activation in response to hypertrophic stimulation (Figure 4.3A, upper panel). The total level of ERK was unaffected, confirming that ERK activity, and not abundance, is influenced by FCS (Figure 4.3A, lower panel). Figure 4.3B (upper panel) shows that c-Myc is also induced by hypertrophic stimulation. The constant level of total ERK (Figure 4.3B, lower panel) indicates equal protein loading in both lanes. These observations are consistent with previous studies, which have shown ERK activation and c-Myc induction to be features of the hypertrophic growth response (Bogoyevitch et al, 1993; Bogoyevitch et al, 1994; Izumo et al, 1988; Sadoshima and Izumo, 1993a; Sadoshima et al, 1995; Starsken et al, 1986), and support the proposed involvement of these proteins in regulating pol III transcription in cardiomyocytes.

Mitogenic stimulation of proliferating cells induces the phosphorylation of RB at multiple sites (Hulleman and Boonstra, 2001; Kaelin, 1999; Weinberg, 1995). This is a fundamental requirement for cell cycle progression (Hulleman and Boonstra, 2001; Kaelin, 1999; Weinberg, 1995). One of the consequences of RB hyperphosphorylation is the release of TFIIIB, which relieves pol III transcriptional repression (Scott et al, 2001). Although cardiomyocytes do not proliferate, RB phosphorylation is thought to occur in response to hypertrophic stimulation (Sadoshima et al, 1997). This was confirmed in the present study: Figure 4.3C shows that hypertrophic stimulation leads to the phosphorylation of

at least three separate RB residues (serine 795, serine 780 and threonine 826), known to be important for RB function (Connell-Crowley et al, 1997; Kitagawa et al, 1996; Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997). Hypertrophic stimulation has little effect on the total level of RB.

Hyperphosphorylation can reduce the electrophoretic mobility of RB. Although this was not apparent when lysates were resolved on 7.8% SDS-polyacrylamide gels (Figure 4.3C, second panel from bottom), electrophoresis of protein samples using gels with a lower percentage acrylamide (6%) revealed that a portion of RB, derived from serum-exposed cells, displayed a mobility shift (Figure 4.3C, bottom panel). This provides further evidence to suggest that RB becomes hyperphosphorylated in response to hypertrophic stimulation. Therefore, this may contribute to the upregulation of pol III transcription during hypertrophy.

4.2.3 Hypertrophic stimulation of cardiomyocytes specifically induces Brf1 expression

In dividing cells, pol III transcription is regulated primarily by proteins that interact with the transcription machinery, rather than through alterations in transcription factor abundance. To test whether the same were likely to be true in cardiomyocytes, Western blots were carried out to compare the effects of FCS-stimulation on the levels of TFIIIB and TFIIIC subunits in cultured fibroblast cells and cardiomyocytes. As expected, the levels of all the TFIIIB and TFIIIC subunits tested (Brf1, Bdp1, TBP and TFIIIC110) were unchanged following FCS-stimulation of a rat fibroblast cell line (Figure 4.4A). In contrast, there was

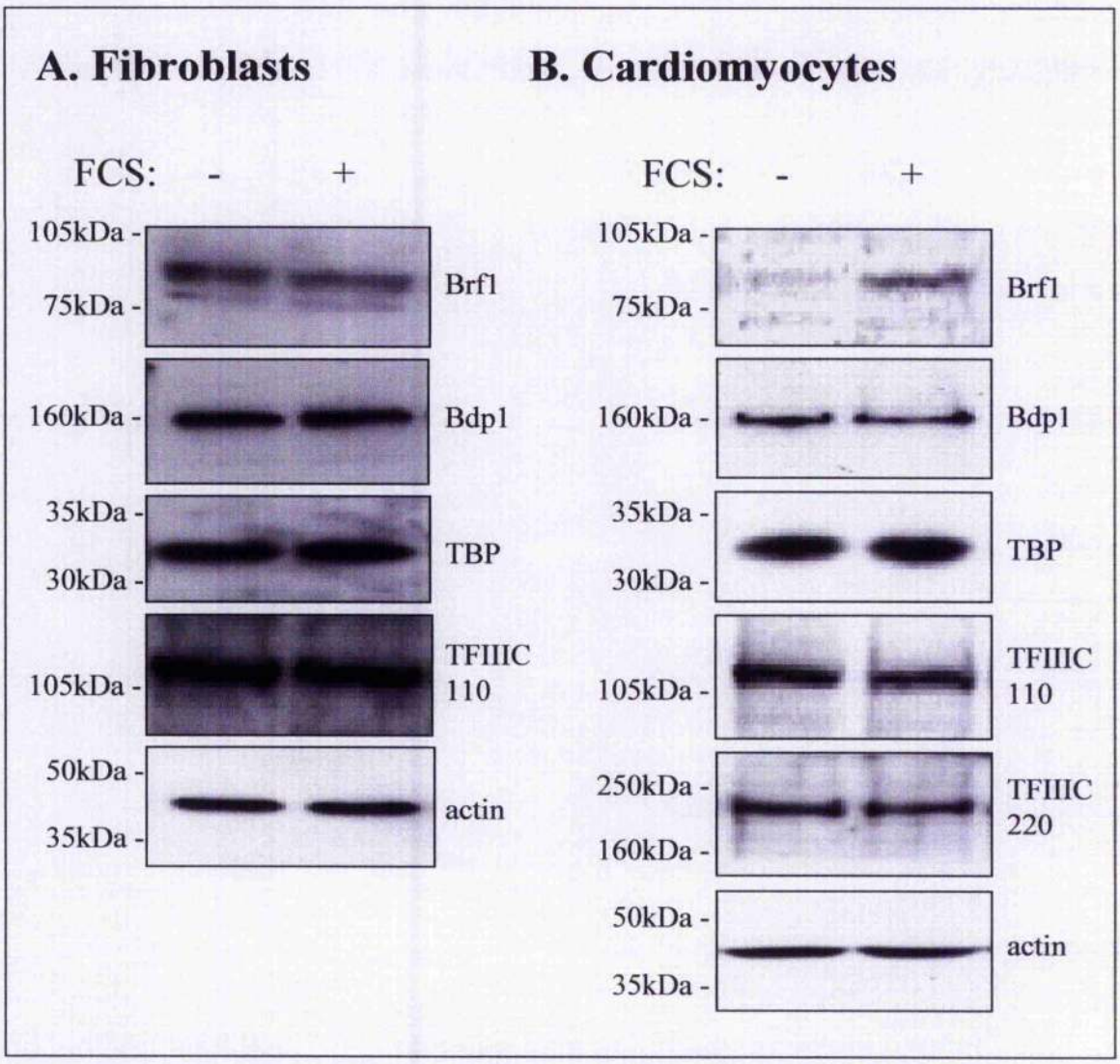


Figure 4.4: Brf1 protein levels specifically increase in cardiomyocytes, but not fibroblasts, in response to growth stimulation. Whole cell lysates were prepared from cultured rat 1A fibroblasts (A) or cardiomyocytes (B) that had been serum-starved for 24 hours, then either maintained in serum-free medium or exposed to 10% FCS for 16 hours. 75 μg of protein were then resolved by SDS-PAGE. Western blotting was performed with antibodies against the proteins indicated.

a significant induction of Brf1 expression in response to the hypertrophic stimulation of cardiomyocytes (Figure 4.4B). This effect is specific to Brf1, as the levels of the other TFIIIB subunits, Bdp1 and TBP, and the TFIIIC subunits, TFIIIC110 and TFIIIC220, remained unchanged. The constant level of actin in Figures 4.4A and B indicate that protein was loaded equally in both cases. Potentially, increased Brf1 expression may represent a mechanism for increasing TFIIIB activity in cardiomyocytes, distinct from mechanisms operating during cellular proliferation.

4.2.4 Comparison of pol III transcriptional activation, and changes in potential regulators of pol III transcription, over time

Thus, hypertrophic growth accompanies the activation of ERK and c-Myc, the hyperphosphorylation of RB, and the induction of Brf1. To gain further insights into the role these proteins may play in upregulating pol III transcription during hypertrophy, the kinetics of pol III activation and the induction or phosphorylation of each of these potential regulators were compared. Cultured cardiomyocytes were serum-starved for 24 hours then exposed to FCS for various lengths of time (ranging from 0 to 48 hours). Total cellular RNA or protein were then prepared and analysed by Northern or Western blotting, respectively. To assess the induction of pol III transcription over this timecourse, B2 levels were measured by Northern blotting (Figure 4.5). This indicates that pol III transcription increases significantly, as early as 2 hours following hypertrophic stimulation, and reaches maximum levels between 12 and 24 hours.

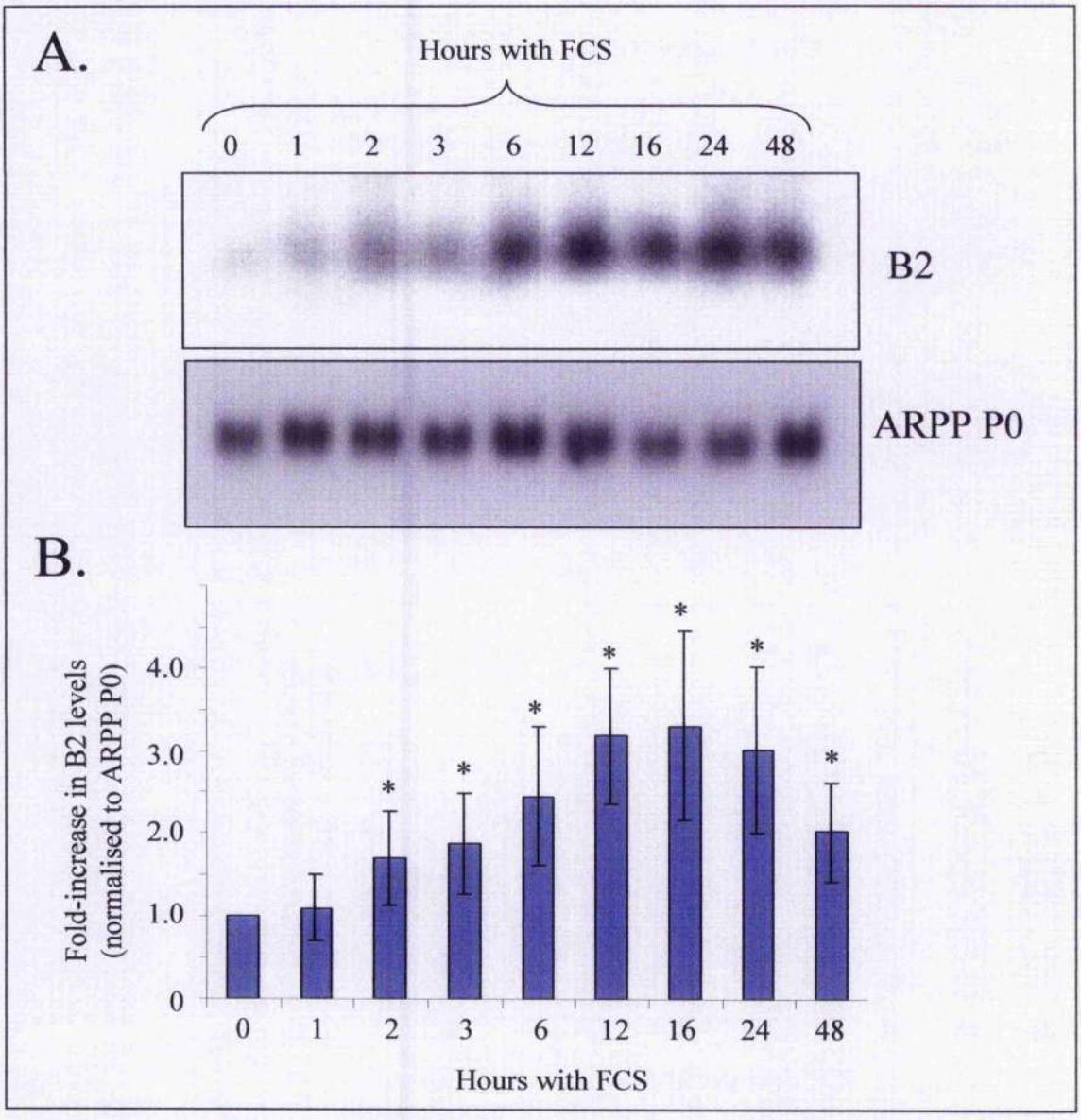
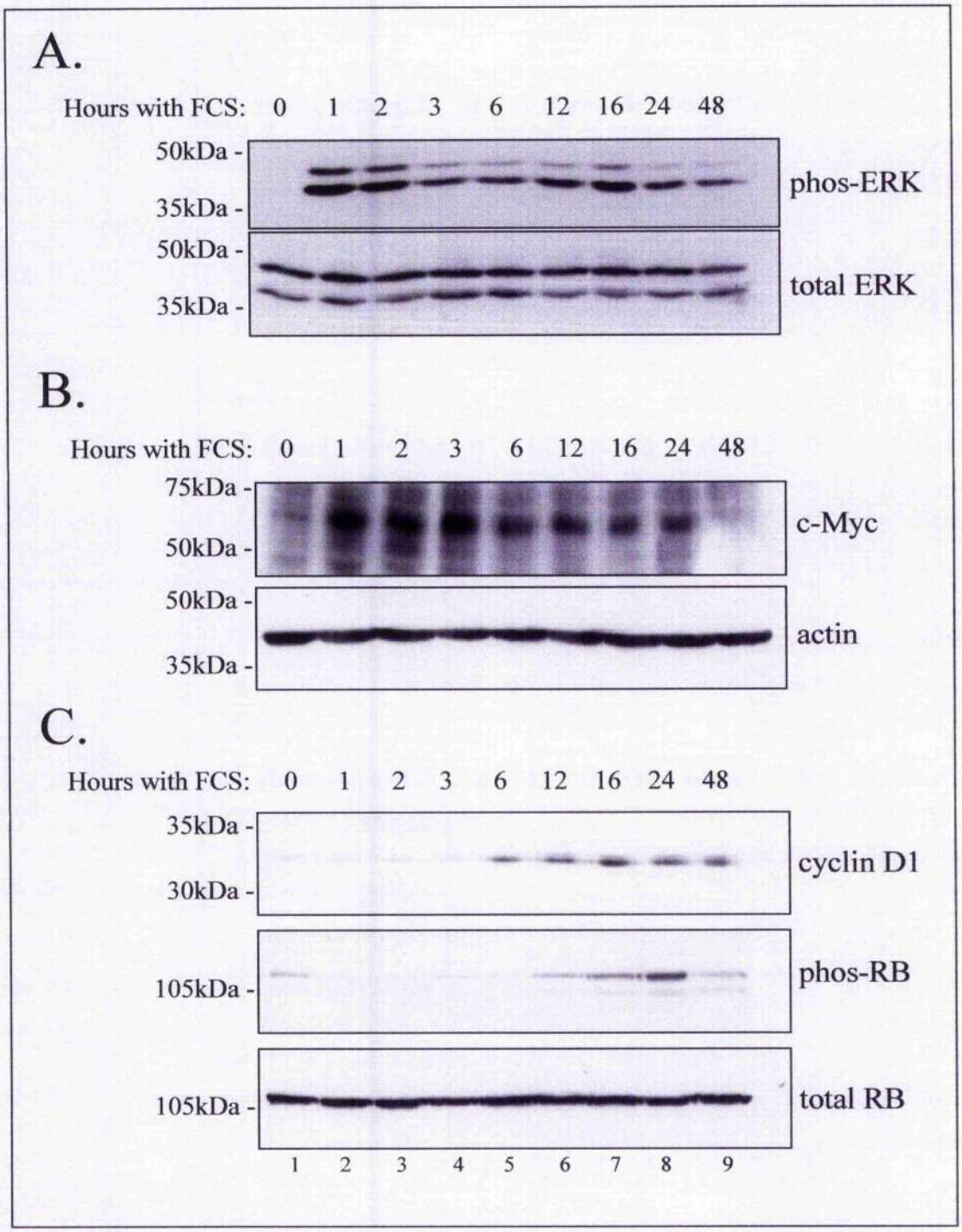


Figure 4.5: Changes in pol III transcription over time following the hypertrophic stimulation of cultured cardiomyocytes. Total RNA was extracted from cardiomyocytes that had been cultured in serum-free medium for 24 hours, then stimulated with 10% FCS for the times indicated (0 to 48 hours). 10 μg of this RNA was analysed by Northern blotting. A. Example of such a Northern blot. The upper panel shows the blot probed for B2, and the lower panel shows the same blot stripped and re-probed for ARPP P0. B. The levels of B2 and ARPP P0 in each sample were quantified using densitometry, then B2 normalised against ARPP P0. This was done for 4 separate experiments. The average fold increases obtained, along with standard deviations from the means, are represented in the graph (*significantly different from control, $p<0.05$).

B2 levels then decline again between 24 and 48 hours. The level of the control mRNA, ARPP P0, remains unchanged.

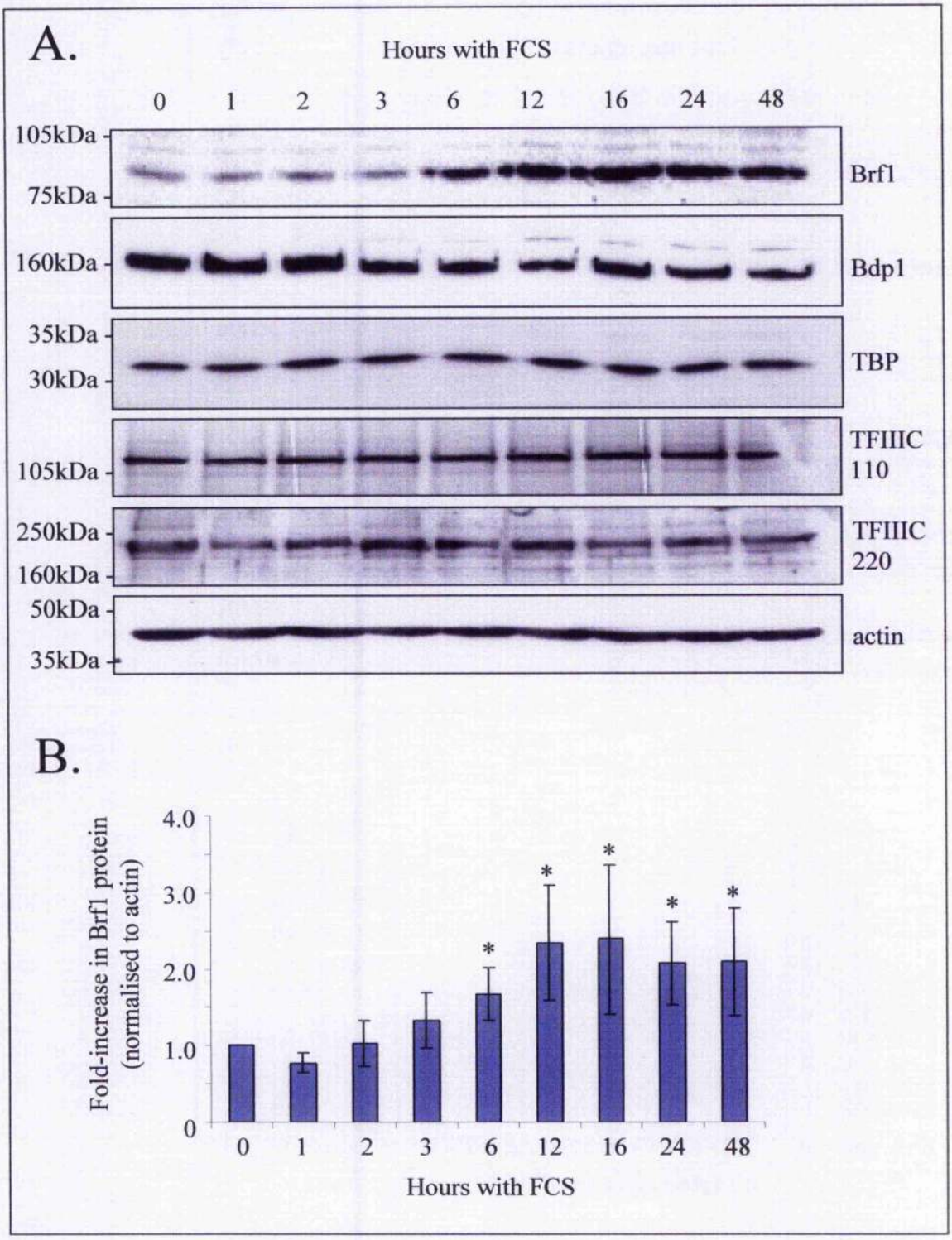
Figure 4.6 demonstrates how known regulators of TFIIIB are affected over this same time period. Both ERK and c-Myc are activated after 1 hour of hypertrophic stimulation, thus preceding the initial increase in pol III transcription (Figures 4.6A and B). Therefore, these proteins could potentially be involved in the early induction of transcription by pol III. RB phosphorylation occurs 6 to 12 hours after hypertrophic stimulation (Figure 4.6C). Consequently, this is unlikely to contribute to the initial activation of pol III transcription during hypertrophy. However, RB phosphorylation does coincide with the peak of B2 levels, suggesting that release of TFIIIB from RB may be required for maximum pol III transcription. In addition, the level of phosphorylated RB decreases between 24 and 48 hours. Likewise, c-Myc levels decline towards the end of the timecourse. These effects may at least partly account for the reduction in pol III transcripts at this time. As expected, RB phosphorylation was preceded by the induction of cyclin D1 (Figure 4.6C, top panel), which is known to be directly responsive to growth factors, in both proliferating cells and cardiomyocytes, and is required for CDK4/6 activity (Hulleman and Boonstra, 2001; Nozato et al, 2001; Sadoshima et al, 1997; Sherr, 1996; Tamamori et al, 1998). These cyclin D-dependent kinases are responsible for the initial RB phosphorylation events that occur following the mitogenic stimulation of dividing cells (Hulleman and Boonstra, 2001; Sherr, 1996). Levels of total ERK, actin and total RB (Figures 4.6A, B and C, respectively) were constant throughout the timecourse.

Figure 4.6: Changes in ERK, c-Myc and RB over time following the hypertrophic stimulation of cultured cardiomyocytes. Whole cell lysates were prepared from cardiomyocytes cultured in serum-free medium for 24 hours, then stimulated with 10% FCS for the times indicated (0 to 48 hours). Western blotting was performed with antibodies against the proteins specified. A. ERK activation precedes the initial induction of pol III transcription. B. c-Myc induction precedes the initial activation of pol III transcription. The anti-c-Myc antibody C8 was used here. C. RB phosphorylation coincides with the highest level of pol III transcription. RB phosphorylation was measured here using the antibody that specifically detects RB phosphorylated on thr 826. The anti-RB antibody C15 was used to measure the level of total RB.


A.
Hours with FCS: 0 1 2 3 6 12 16 24 48
50kDa -
35kDa -
phos-ERK
50kDa -
35kDa -
total ERK
B.
Hours with FCS: 0 1 2 3 6 12 16 24 48
75kDa -
50kDa -
c-Myc
50kDa -
35kDa -
actin
C.
Hours with FCS: 0 1 2 3 6 12 16 24 48
35kDa -
30kDa -
cyclin D1
105kDa -
phos-RB
105kDa -
total RB
1 2 3 4 5 6 7 8 9

Alterations in TFIIIB and TFIIIC subunit levels over time were also examined by Western blotting (Figure 4.7). As expected, Bdp1, TBP, TFIIIC110, TFIIIC220 and actin levels do not change. In contrast, Brf1 levels increase significantly after 3 to 6 hours of exposure to FCS. Maximum levels of Brf1 (approximately 2.4-fold higher than control cells) are attained after 16 hours of hypertrophic stimulation. As with RB phosphorylation, this correlates with the highest level of pol III transcription.

Figure 4.7: Changes in TFIIIB and TFIIIC subunit levels over time following the hypertrophic stimulation of cultured cardiomyocytes. Whole cell lysates were prepared from cardiomyocytes cultured in serum-free medium for 24 hours, then stimulated with 10% FCS for the times indicated (0 to 48 hours). Proteins were resolved by SDS-PAGE. A. Western blotting was performed with antibodies against the proteins indicated. B. Using densitometry, the level of Brf1 was quantified at each timepoint and normalised to actin. This was done for 3 separate experiments. The average fold increases obtained, along with standard deviations from the means, are represented in the graph (*significantly different from control, $p<0.05$).


A.
Hours with FCS
0 1 2 3 6 12 16 24 48
105kDa
75kDa
Brf1
160kDa
Bdp1
35kDa
30kDa
TBP
105kDa
TFIIIC 110
250kDa
160kDa
TFIIIC 220
50kDa
35kDa
actin
B.
Fold-increase in Brf1 protein (normalised to actin)
4.0
3.0
2.0
1.0
0
0 1 2 3 6 12 16 24 48
Hours with FCS

4.3 Discussion

Transcription initiation complex assembly on class III genes is enhanced by hypertrophic stimulation of cardiomyocytes, as demonstrated by ChIP analyses. This appears to be regulated at the level of TFIIIB recruitment. The activities of known TFIIIB regulators were investigated in the current chapter. This demonstrated the activation of ERK and c-Myc, and the inactivation of the TFIIIB repressor RB in response to hypertrophic stimulation. In addition, the Brf1 subunit of TFIIIB is specifically induced during hypertrophy. Temporal examination of these changes revealed that ERK and c-Myc induction precede the initial activation of pol III transcription, whereas RB phosphorylation and Brf1 induction correlate with the later, maximum level of pol III transcripts. Thus, this chapter displays a variety of mechanisms potentially involved in the activation of pol III transcription during cardiomyocyte hypertrophy, and provides a basis for the further investigation presented in subsequent chapters.

Serum-induced changes in class III gene promoter occupancy have not previously been documented in mammalian cells *in vivo*. The data presented here demonstrate that enhanced TFIIIB recruitment coincides with increased pol III binding, and the activation of pol III transcription. Although this scenario has not previously been reported to accompany the serum stimulation of pol III transcription, decreases in TFIIIB, and not TFIIIC, promoter occupancy correlate with the reduction in pol III transcription associated with ERK inhibition (Felton-Edkins et al, 2003a), p53 induction (Crighton et al, 2003) and mitosis (Fairley et al, 2003) in mammals, and the transition from growth to stationery phase in yeast (Harismendy et al, 2003). This indicates that altered TFIIIB recruitment is a

common means of regulating pol III transcription, and is consistent with the well-recognised role of TFIIIB as a target for numerous regulatory mechanisms.

Of these known control mechanisms, ERK, c-Myc and RB were considered likely candidates for the regulation of pol III transcription in cardiomyocytes. This was based on the fact that these proteins have been implicated in mediating hypertrophic growth (Bueno and Molkentin, 2002; MacLellan et al, unpublished results; Nozato et al, 2001; Sadoshima et al, 1997; Simpson, 1988; Tamamori et al, 1998; Xiao et al, 2001). Consistent with this, the current study demonstrated the hypertrophy-associated activation of ERK and c-Myc, and the phosphorylation of RB. ERK activation has been reported to occur after only 1 minute of hypertrophic stimulation (Clerk et al, 1994), and can be sustained for up to 48 hours (Iijima et al, 2002). Although such a rapid induction was not investigated here, ERK was found to be activated at the earliest timepoint tested. In addition, c-Myc induction and RB phosphorylation occurred with kinetics similar to those previously reported (Izumo et al, 1988; Sadoshima et al, 1997; Starsken et al, 1986). These molecular changes coincided with distinct phases of pol III transcriptional activation. Therefore, the significance of each of these proteins in regulating pol III transcription in cardiomyocytes was investigated further, as detailed in Chapter 5.

Although RB phosphorylation is significantly higher after 16 hours of hypertrophic stimulation than in the absence of FCS (Figure 4.2C), analysis of RB phosphorylation over time revealed an unexpectedly high level of phosphorylated RB in unstimulated cells compared to cells stimulated with FCS

for 1 to 6 hours (Figure 4.5C, compare lane 1 with lanes 2-5). The significance of this surprising observation is unclear. However, it was a consistent feature of several timecourses, and may indicate that initially, basal CDK activity is inhibited by hypertrophic stimulation. Interestingly, the association of CDK activity with a D-type cyclin has been reported to decrease during the first 6 hours of hypertrophic stimulation with FCS (Sadoshima et al, 1997). RB phosphorylation was monitored over time using an antibody that specifically detects phosphorylation of the cyclin D-dependent kinase target site, threonine 826 (Zarkowska and Mittnacht, 1997). Possibly, the decrease in phosphorylation after 1 hour of FCS exposure may be specific to this particular residue. Whatever the explanation for the initial decrease in RB phosphorylation, it did not accompany a decline in pol III transcription.

Stimulation of undifferentiated fibroblast cells with FCS increases pol III transcription (Felton-Edkins et al, 2003a; Johnson et al, 1974; Mauck and Green, 1974; Scott et al, 2001), but does not affect the levels of TFIIIB or TFIIIC, as shown in this chapter (Figure 4.4A) and by other investigators (Felton-Edkins et al, 2003a; Johnston et al, 2002; Scott et al, 2001; Winter et al, 2000). This indicates that the basal levels of these factors are sufficient to permit increases in transcription, in response to growth stimulation. As previously discussed, this is achieved through regulating transcription factor activity, rather than abundance. Cardiomyocytes are terminally differentiated cells. Differentiation of F9 embryonal carcinoma cells has been shown to accompany a dramatic decrease in the rate of pol III transcription, specifically caused by a reduction in TFIIIB activity (White et al, 1989). This decrease in TFIIIB activity can be at least partly

attributed to a decline in Brf1 levels (Alzuherri and White, 1998). Thus, potentially, the low level of Brf1 in unstimulated cardiomyocytes (Figures 4.4 and 4.7) may reflect the differentiated nature of these cells, and could restrict the formation of functional TFIIIB complexes, and thereby restrain pol III transcription. If this were the case, an increase in the level of Brf1 would be required to enhance pol III transcription in cardiomyocytes, in contrast to the situation in undifferentiated fibroblasts. In support of this proposed mechanism, Brf1 levels do increase in response to the hypertrophic stimulation of cardiomyocytes (Figures 4.4 and 4.7). The relevance of this to the hypertrophy-associated activation of pol III transcription is addressed in Chapter 6.

Although hypertrophic stimuli do not induce cardiomyocyte proliferation, many of the responses they do initiate also occur in proliferating cells in response to mitogens and growth factors. Some of these changes, including RB phosphorylation and the activation of ERK and c-Myc, play important roles in the regulation of pol III transcription during cellular proliferation. It is possible that these same mechanisms are used to regulate pol III transcription in cardiomyocytes, in order to facilitate the rapid growth demands of these non-dividing cells during hypertrophy. Distinct regulatory mechanisms may also contribute to the control of pol III transcription in fibroblasts and cardiomyocytes, possibly reflecting differences in their states of differentiation and proliferative capacity. For example, hypertrophic stimulation may increase pol III transcription in cardiomyocytes through the induction of Brf1. The following chapters address these possibilities.

CHAPTER 5
Regulation of pol III transcription by ERK, c-Myc and RB in cardiomyocytes

5.1 Introduction

The mechanisms regulating pol III transcription in terminally differentiated cardiomyocytes are unknown. The data presented in Chapter 4 are consistent with a role for ERK, c-Myc and RB in this regulation. The aim of this chapter was to test the hypothesis that these proteins, known to regulate pol III transcription in proliferating cells, are also involved in controlling the hypertrophy-associated increase in pol III transcription in cardiomyocytes. The potential involvement of each of these molecules is summarised in Figure 5.1.

5.1.1 ERK and cardiomyocyte hypertrophy

One of the most intensely studied groups of signalling pathways, activated by hypertrophic stimuli, are the MAP kinase cascades. The MAP kinase superfamily consists of several subfamilies, the best characterised of which are the ERKs (1 and 2), the c-Jun N-terminal kinases (JNKs) and the p38-MAP kinases (p38s) (Chang and Karin, 2001; Garrington and Johnson, 1999; Wilkinson and Millar, 2000). The latter two subfamilies are also known as the stress-responsive MAP kinases. Each of these subfamilies is subject to regulation in the myocardium, as illustrated in Figure 5.2, and the complex mechanisms responsible for this regulation are beginning to be unravelled (reviewed in Bueno and Molkentin, 2002; Clerk and Sugden, 1999; Molkentin and Dorn II, 2001; Sugden, 1999; Sugden and Clerk, 1998).

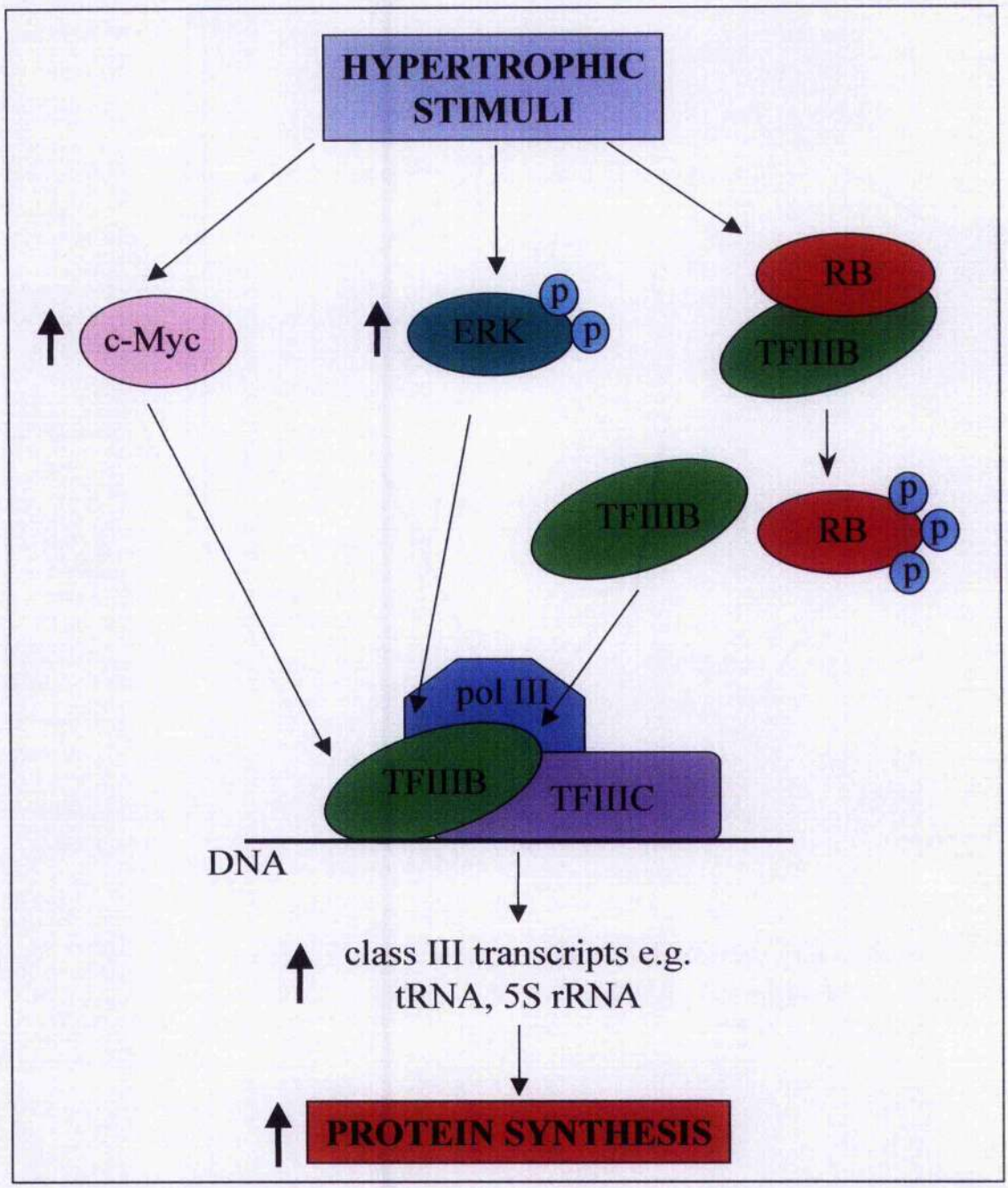


Figure 5.1: ERK, c-Myc and RB have the potential to regulate pol III transcription in cardiomyocytes. Hypertrophic stimulation of cardiomyocytes leads to the activation of ERK and c-Myc, and the phosphorylation of RB. In proliferating cells, each of these proteins regulates pol III transcription by targeting TFIIIB. Both ERK and c-Myc activate pol III transcription, whereas RB represses it. Hyperphosphorylation of RB relieves this repression. Potentially, these same mechanisms may participate in controlling pol III transcription in cardiomyocytes, in order to regulate protein synthesis.

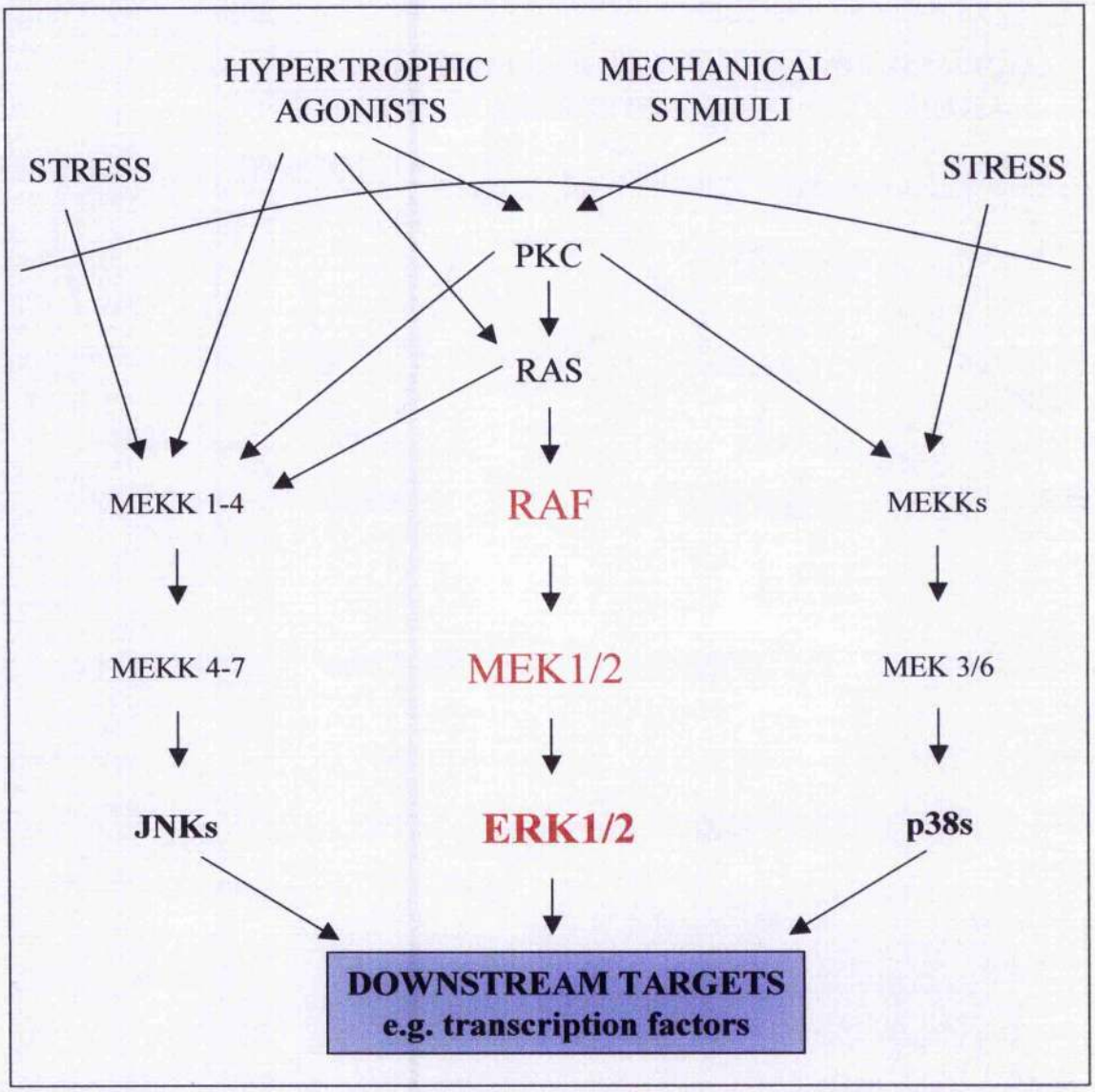


Figure 5.2: Summary of MAP kinase cascade activation in cardiomyocytes. MAP kinase signalling pathways are activated by numerous hypertrophic agonists and stress stimuli in cardiomyocytes. Initial signals are transduced through mechanisms involving molecules such as PKC and ras, leading to the activation of MAP kinase kinase kinase (MEKK) factors, which in turn activate MAP kinase kinase (MEK) enzymes. This culminates with the activation of the MAP kinase effectors JNKs, ERKs and p38s. These kinases subsequently phosphorylate a variety of downstream targets, including nuclear transcription factors. Each of these pathways has a distinct set of targets, but they may also share some common targets. Proteins specific to the ERK pathway are highlighted in red.

The role of ERK in mediating cardiomyocyte hypertrophy has been particularly well studied. Many hypertrophic stimuli, including FCS, ET-1, PE and mechanical stress, activate the ERK pathway in cultured cardiomyocytes (Bogoyevitch, et al, 1993; Bogoyevitch, et al, 1994; Sadoshima and Izumo, 1993a; Sadoshima et al, 1995). Signalling through both PKC and the small G-protein ras have been implicated in this activation (Bogoyevitch, et al, 1993; Bogoyevitch, et al, 1994; Braz et al, 2002; Clerk, et al, 1994; Iijima et al, 2002; Pan et al, 2004; Sugden and Clerk, 2000; Thorburn, 1994). Several studies have demonstrated the relevance of this cascade to hypertrophy, by showing that ERK activation is both necessary and sufficient for the induction of many of the features of the hypertrophic response in culture, including an increase in protein synthesis, enhanced sarcomere organisation and foetal gene re-expression (Bueno et al, 2000; Gillespie-Brown et al, 1995; Glennon, et al, 1996; Ueyama et al, 2000; Wang and Proud, 2002b; Yue et al, 2000).

ERK is also activated by hypertrophic stimulation of the heart (Harris et al, 2004; Iijima et al, 2002; Takeishi et al, 2001). As in cultured cells, the importance of this activation has been clearly shown. For example, transgenic mice expressing, in the myocardium, a constitutively activated form of MEK1, a specific upstream activator of ERK, display a hypertrophic phenotype. This is characterised by enlarged hearts and transcriptional changes that typically accompany hypertrophy (Bueno et al, 2000). Furthermore, cardiac-specific expression of a dominant negative form of Raf, which specifically inhibits ERK activation, blocks hypertrophic growth in response to pressure overload in mice (Harris et al, 2004). ERK activation has also been shown to correlate with hypertrophic

severity in failing human hearts: a therapeutic intervention that reduces the level of active ERK also reduces cardiomyocyte size (Flesch et al, 2001). Studies such as these indicate a central role for the Raf/MEK/ERK pathway in determining the physiological hypertrophic state.

Thus, the ERK pathway appears to be a critical regulator of cardiomyocyte hypertrophy, both in culture and in the heart. ERK mediates its effects on cell function by phosphorylating a range of downstream targets (Chang and Karin, 2001; Wilkinson and Millar, 2000). These targets vary depending on the cell type and initiating stimulus. In cardiomyocytes, several substrates for ERK have been identified. These include nuclear transcription factors, such as the tissue-specific factor GATA4, and the transcriptional coactivators CBP and p300 (Babu et al, 2000; Gusterson et al, 2002; Liang et al, 2001; Morimoto et al, 2000). By phosphorylating such factors, the ERK pathway is thought to contribute to the characteristic re-programming of mRNA production that occurs in cardiomyocytes during hypertrophy.

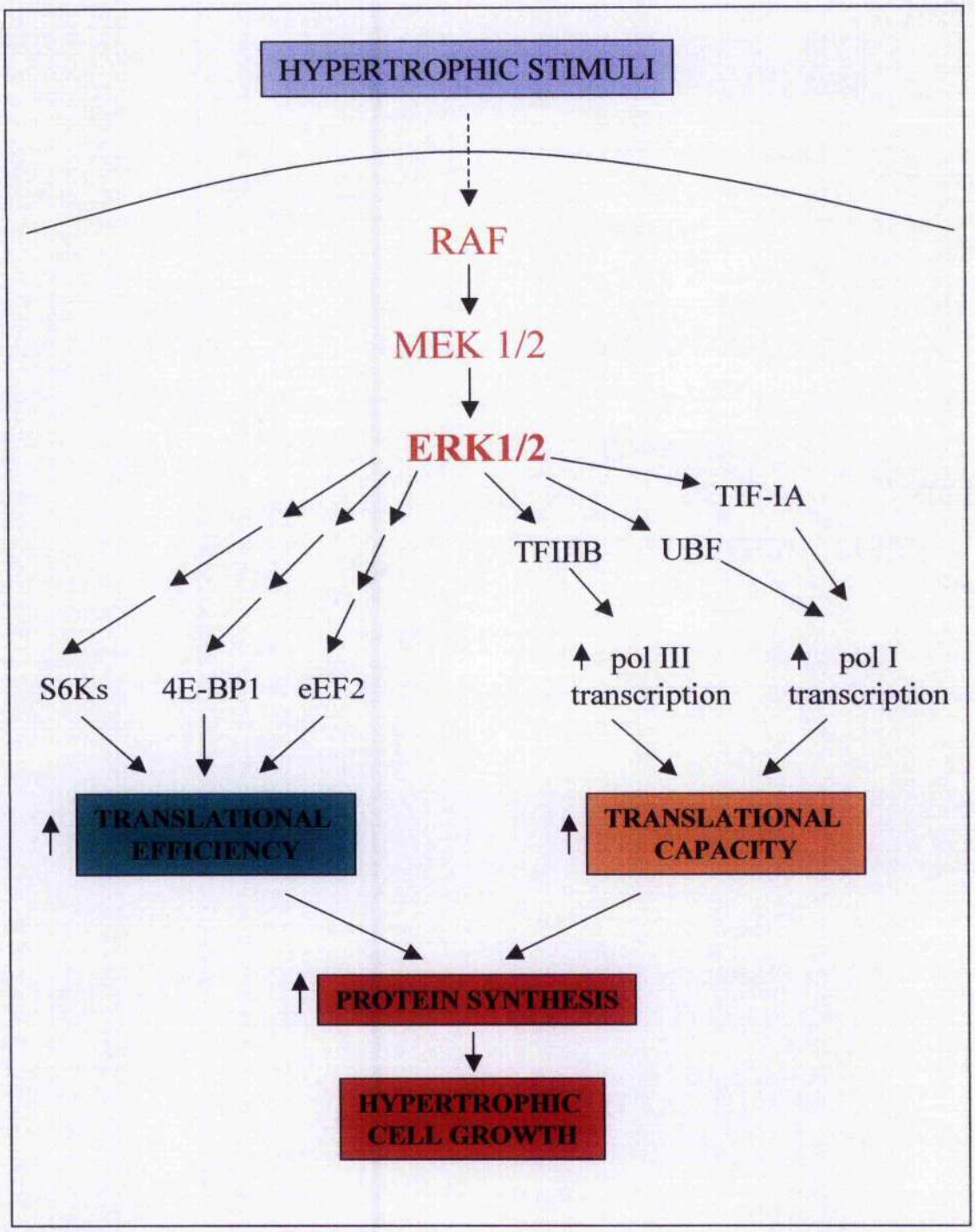
In addition to mediating typical hypertrophy-associated changes in class II gene expression, the Raf/MEK/ERK cascade also induces protein synthesis in cardiomyocytes (Bueno et al, 2001; Uemeya et al, 2000; Wang and Proud, 2002b; Yue et al, 2000). However, the mechanisms by which ERK regulates this biosynthesis are poorly understood. Several recent studies using cultured cardiomyocytes have identified downstream targets of the ERK pathway that may contribute to this ability to stimulate protein synthesis. These include the ribosomal protein S6 kinases (S6Ks), eIF4E-binding protein 1 (4E-BP1) and the

translation elongation factor eEF2 (Iijima et al, 2002; Wang and Proud, 2002a, b; Wang et al, 2001). Each of these proteins is involved in regulating translational efficiency, and consequently, their activities are likely to influence the rate of protein synthesis. However, ERK activation is sufficient to induce sustained hypertrophic growth (Bueno et al, 2000), which, as discussed in Chapter 3, requires an increase in translational capacity. Therefore, it is highly probable that ERK will also target additional factors in order to increase the level of components of the protein synthetic machinery, such as rRNAs and tRNAs. In proliferating, immortalised cell lines, ERK induces rRNA and tRNA production by stimulating transcription by pols I and III (Felton-Edkins et al, 2003a; Stefanovsky, 2001; Zhao et al, 2003). ERK achieves this activation by phosphorylating the pol I-specific transcription factors UBF and TIF-1A, and the pol III-specific factor TFIIIB (Felton-Edkins et al, 2003a; Stefanovsky, 2001; Zhao et al, 2003). This induction of class I and III gene expression by ERK may also occur in cardiomyocytes, and would provide a mechanistic link between ERK activation and the increased protein synthesis that underlies hypertrophic growth. The potential mechanisms by which ERK could induce protein synthesis in cardiomyocytes are summarised in Figure 5.3. The role of ERK in pol III transcriptional activation in cardiomyocytes is addressed in this chapter.

5.1.2 c-Myc and cardiomyocyte hypertrophy

The c-Myc transcription factor is a member of a family of proteins, also including N-Myc, L-Myc, B-Myc and S-Myc (Dang, 1999). The Myc proteins play critical roles in the control of numerous cellular functions, including cell

Figure 5.3: Mechanisms potentially involved in the activation of protein synthesis by ERK during cardiomyocyte hypertrophy. ERK has the potential to increase both translational efficiency and capacity. In response to hypertrophic stimulation, ERK-dependent mechanisms modulate the activities of S6Ks, 4E-BP1 and eEF2. This is likely to increase cardiomyocyte translational efficiency. TFIIIB, UBF and TIF-1A are directly phosphorylated by ERK in proliferating cells, and this leads to the induction of pol I and III transcription, as indicated. This may also occur in cardiomyocytes, and hence facilitate an increase in translational capacity. Thus, several downstream targets of the ERK pathway are likely to mediate the increased protein synthesis required for cardiomyocyte hypertrophic growth.


HYPERTROPHIC STIMULI
RAF
MEK 1/2
ERK1/2
TIF-IA
TFIIIB
UBF
S6Ks
4E-BP1
eEF2
pol III transcription
pol I transcription
TRANSLATIONAL EFFICIENCY
TRANSLATIONAL CAPACITY
PROTEIN SYNTHESIS
HYPERTROPHIC CELL GROWTH

growth, proliferation, apoptosis and differentiation (Dang, 1999; Lüscher, 2001; Schmidt, 1999; Zhou and Hurlin, 2001). These profound physiological effects are likely to reflect the ability of Myc to directly modulate the expression of multiple target genes (Coller, 2000; Dang, 1999). This is mediated by two domains: a C-terminal basic/helix-loop-helix/leucine zipper (b/HLH/z) DNA-binding domain, and an N-terminal transactivation domain (TAD) (Dang, 1999; Lüscher, 2001). Many genes activated by c-Myc have a consensus c-Myc binding site (5'-CACGTG-3'), known as an E-box (Dang, 1999; Lüscher, 2001). In order to bind these sites, c-Myc must heterodimerise with its binding partner Max, via the C-terminal b/HLH/z domain (Dang, 1999; Lüscher, 2001). c-Myc then activates transcription. For at least some target genes, this occurs through the TAD-mediated recruitment of various chromatin remodelling activities (Amati et al, 2001). However, some genes are directly repressed by c-Myc in an E-box-independent manner (Amati et al, 2001; Lüscher, 2001).

c-Myc activates the transcription of an array of genes whose products promote cell growth and division, while repressing the expression of negative regulators of these processes (Coller et al, 2000; Dang, 1999; Guo et al, 2000; O'Connell et al, 2003). Consequently, abnormal c-Myc expression is a common feature of human cancers. As a result, intensive research efforts have focused on delineating the mechanisms of c-Myc regulation and function. In non-tumour cells, the level of c-Myc is stringently controlled: c-Myc levels respond to mitogens and growth factors and are high in rapidly proliferating cells, but dramatically decline as cells differentiate (Grandori et al, 2000; Lüscher, 2001; Zhou and Hurlin, 2001). This regulation of c-Myc expression appears very

complex, involving transcriptional, post-transcriptional, translational and post-translational controls (Dean et al, 1985; Jones and Cole, 1987; Kelly et al, 1983; Lüscher, 2001; Sears et al, 1999; West et al, 1998). The mechanisms regulating these processes are ill-defined; however, several signalling cascades, including the Ras/Raf/ERK pathway, have been implicated in certain aspects of this control (Cheng et al, 1999; Kerkhoff and Rapp, 1998; Sears et al, 1999; Sears et al, 2000).

Thus, c-Myc was initially characterised as a protein that promotes cellular proliferation and oncogenesis. An important prerequisite for cell division is attainment of a critical mass (Conlon and Raff, 1999; Schmidt, 1999). Accordingly, c-Myc is thought to induce proliferation not only by directing the expression of proteins involved in cell cycle progression and DNA synthesis, such as cyclins, CDKs, dihydrofolate reductase and thymidine kinase, but also by influencing the production of factors involved in protein synthesis, including translation factors and proteins involved in ribosome biogenesis (Coller et al, 2000; Dang, 1999; Guo et al, 2000; O'Connell et al, 2003; Schmidt, 1999). In fact, in some instances, c-Myc is now recognised as an important regulator of cell division-independent cell growth (Iritani and Eisenman, 1999; Johnston et al, 1999; Kim et al, 2000; Schuhmacher et al, 1999).

As indicated above, terminally differentiated cells, such as cardiomyocytes, express c-Myc at very low levels, or not at all (Grandori et al, 2000; Komuro et al, 1988; Schneider et al, 1986; Zhou and Hurlin, 2001). However, studies of the molecular changes occurring during cardiomyocyte hypertrophy have revealed

that various hypertrophic stimuli, which do not promote proliferation, rapidly increase c-Myc at the mRNA and protein levels (Izumo et al, 1988; Komuro et al, 1988; Sadoshima et al, 1992; Starsken et al, 1986; Chapter 4, Figure 4.6). The significance of this induction is only beginning to be appreciated, following reports that c-Myc expression is directly involved in promoting hypertrophy in the adult heart (Robbins and Swain, 1992; Xiao et al, 2001). Furthermore, clinical studies have demonstrated a correlation between the level of c-Myc expression and cardiomyocyte size in hypertrophic human hearts (Kai et al, 1998).

The molecular events downstream of c-Myc induction in cardiomyocytes, which facilitate hypertrophic growth, have not been characterised. Candidate target genes include those encoding ribosomal proteins (L3, L23 and L35) and other proteins involved in ribosome biogenesis (nucleolin and nucleophosmin) (Xiao et al, 2001). Expression of these genes accompanies c-Myc activation in the adult myocardium, potentially contributing to an increase in protein synthesis (Xiao et al, 2001). Recent additions to the list of growth-promoting genes targeted by c-Myc in proliferating cells are the pol III-transcribed tRNA and 5S rRNA genes (Felton-Edkins et al, 2003b; Gomez-Roman et al, 2003). Although these genes do not have E-box sequences, they are bound and activated directly by c-Myc *in vivo* (Felton-Edkins et al, 2003b; Gomez-Roman et al, 2003). This is thought to be mediated through protein-protein interactions between c-Myc and TFIIIB (Gomez-Roman et al, 2003). Experiments described in this chapter aimed to determine whether c-Myc could also activate class III gene expression in

cardiomyocytes, which would likely contribute to the ability of c-Myc to stimulate cardiomyocyte growth.

5.1.3 Cell cycle proteins and cardiomyocyte hypertrophy

In proliferating cells, mitogenic signals activate transduction cascades involving various proteins, including ERK and c-Myc, which regulate components of the cell cycle machinery (Hulleman and Boonstra, 2001). In this way, extracellular stimuli drive cell cycle progression. Despite the fact that cardiomyocytes become terminally differentiated in the neonate, and largely lose the ability to proliferate, emerging evidence suggests that certain components of this cell cycle machinery may also be involved in regulating the hypertrophic response (Busk et al, 2002; Nozato et al, 2001; Sadoshima et al, 1997; Tamamori-Adachi et al, 2002; Tamamori et al, 1998). For example, cyclin induction and CDK activation have been shown to accompany hypertrophic growth. In particular, several studies have shown that cyclins, normally connected with the G1 phase of the cell cycle (including cyclin D1, 2 and 3), and their associated kinases (CDKs 4 and 6), are induced by hypertrophic stimuli in culture and *in situ* (Busk et al, 2002; Nozato et al, 2001; Sadoshima et al, 1997; Tamamori-Adachi et al, 2002; Tamamori et al, 1998). Investigations into the relevance of these observations infer that these proteins promote hypertrophic growth. For instance, overexpressing cyclin D1 and CDK4 in cultured cardiomyocytes increases protein synthesis and cell size, but does not provoke cell cycle reentry (Tamamori-Adachi et al, 2002). Conversely, inhibition of cyclin D-CDK4/6 complex activity blocks hypertrophic growth in cultured cells and in the heart (Busk et al, 2002; Nozato et al, 2001;

Tamamori et al, 1998). Cyclin D-CDK4 complexes have also been implicated in promoting the hypertrophic growth of terminally differentiated cells in *Drosophila* (Datar et al, 2000), suggesting that this may be a conserved phenomenon.

The most extensively studied target of cyclin D-dependent kinases is the tumour suppressor protein RB (Sherr, 1996; Weinberg, 1995). Hypertrophic stimulation of cultured cardiomyocytes increases the proportion of hyperphosphorylated RB, as shown in Chapter 4 (Figure 4.3) and by other investigators (Nozato et al, 2001; Sadoshima et al, 1997). However, it is not known whether this contributes to the induction of hypertrophic growth by cyclin D-CDK4/6. One of the most important functional consequences of RB phosphorylation in cycling cells is the promotion of S phase entry (Kaelin et al,1999; Sherr, 1996; Weinberg, 1995). This is thought to be achieved through the regulation of class I, II and III gene expression (Graña et al, 1998; White, 1998). Hypophosphorylated RB binds the pol I-, II- and III-specific transcription factors UBF, E2F and TFIIIB, respectively (Dyson, 1998; Hannan et al, 2000; Scott et al, 2001). Phosphorylation of RB by CDKs correlates with the activation of these factors, and the subsequent induction of their target genes (Dyson, 1998; Hannan et al, 2000; Scott et al, 2001). In the case of UBF and TFIIIB, this would mean a general increase in class I and III gene expression, respectively, which is thought to facilitate the increase in cell mass necessary for S phase entry (White, 1998). E2F transcription factor complexes regulate the pol II-dependent transcription of several genes required for DNA synthesis, including DNA polymerase α, thymidine kinase and dihydrofolate reductase (Dyson, 1998; White, 1998). Thus,

such changes in gene expression provide several cellular components essential for the S phase of the cell cycle.

Terminally differentiated cardiomyocytes do not divide. Therefore, to appreciate the full significance of hypertrophy-associated RB phosphorylation, it is important to characterise the downstream effects of this phosphorylation specifically in post-mitotic cardiomyocytes. Potentially, in terminally differentiated cells, RB phosphorylation may selectively induce the expression of genes involved in promoting cell growth, such as those transcribed by pols I and III, without activating those dedicated solely to DNA synthesis.

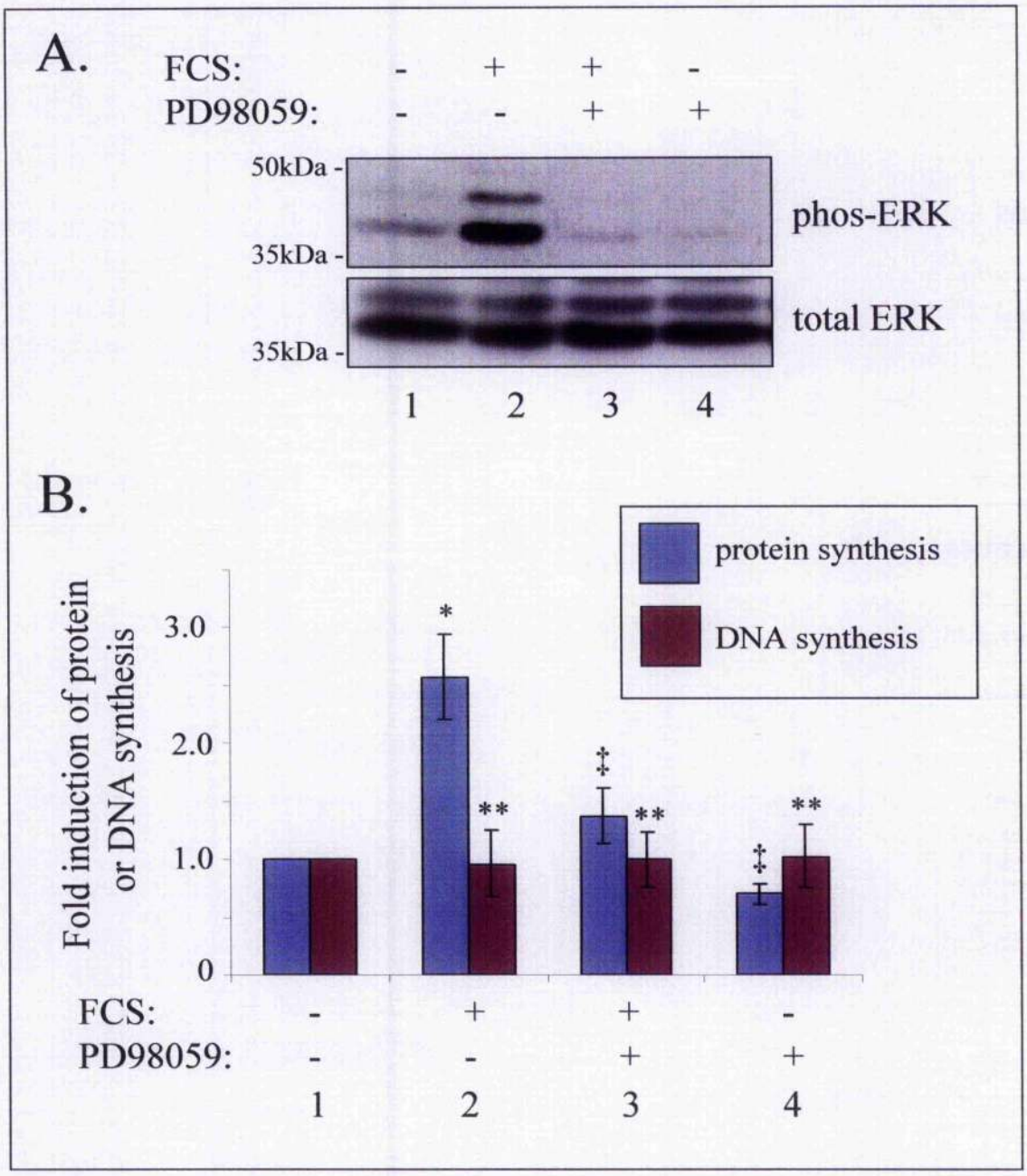
In order to understand the process of myocardial hypertrophy, it is important to delineate the molecular mechanisms contributing to its development. As such, the pathway of events leading from the initial hypertrophic stimulus, to changes in cellular phenotype, must be determined. This chapter aims to establish a link between several proteins shown to be responsive to hypertrophic stimuli, namely ERK, c-Myc and RB, and an increase in protein synthetic capacity through the regulation of pol III transcription.

5.2 Results

5.2.1 Activation of pol III transcription by ERK in cardiomyocytes

As summarised in the introduction to this chapter, a large body of evidence indicates that the Raf/MEK/ERK cascade promotes the induction of hypertrophy in cardiomyocytes. To confirm the involvement of this pathway in the serum-induced hypertrophic response of the primary cells used in this study, a MEK-specific inhibitor (PD98059) was used. Cardiomyocytes were serum-starved for 24 hours then either maintained in serum-free media, or exposed to 10% FCS, for a further 16 hours in the presence of PD98059 (50 μM) or vehicle. ERK activation by FCS is completely blocked in the presence of PD98059 (Figure 5.4A, upper panel), indicating that this compound effectively inhibits MEK1/2 (see Figure 5.2). The total level of ERK is unaffected by these treatments (Figure 5.4A, lower panel). To determine the effects of this inhibition on hypertrophic growth, the rates of protein synthesis were measured (Figure 5.4B). This demonstrated that the characteristic induction of protein synthesis by serum is significantly attenuated in the presence of PD98059 (Figure 5.4B), thus indicating that ERK activation is necessary for the full induction of protein synthesis in response to hypertrophic stimuli. This is in agreement with the findings of several other studies (Ueyama et al, 2000; Wang and Proud, 2002b; Yue et al, 2000), and is consistent with a critical role for the ERK pathway in mediating cardiomyocyte hypertrophy. The rate of DNA synthesis was unaffected, as expected. Although the rate of protein synthesis is markedly reduced when serum-stimulated cardiomyocytes are incubated with PD98059, it is not completely abrogated to basal levels (Figure 5.4B, compare conditions 1

Figure 5.4: ERK inhibition impairs hypertrophic growth in cultured cardiomyocytes. Cardiomyocytes were cultured in the absence of serum for 24 hours, then either maintained in serum-free media for a further 16 hours (conditions 1 and 4), or stimulated with 10% FCS for 16 hours (conditions 2 and 3). 50 μM PD98059 (conditions 3 and 4) or vehicle alone (0.1% DMSO; conditions 1 and 2) were also included, as indicated. A. PD98059 blocks ERK activation. Whole cell lysates were analysed by Western blotting. Representative Western blots, performed with the antibodies specified, are shown. B. PD98059 impairs protein synthesis. Cells were labelled with [^{35}S]methionine/cysteine or [^{3}H]thymidine to determine protein or DNA synthesis rates, respectively. Relative [^{35}S] and [^{3}H] incorporation were assessed by liquid scintillation counting. The fold increases represent the mean of four separate experiments, each with three replicates per condition. The error bars indicate the standard deviation from the mean (*significantly higher than condition 1, $p<0.05$; ‡significantly lower than condition 2, $p<0.05$; **not significantly different from condition 1).

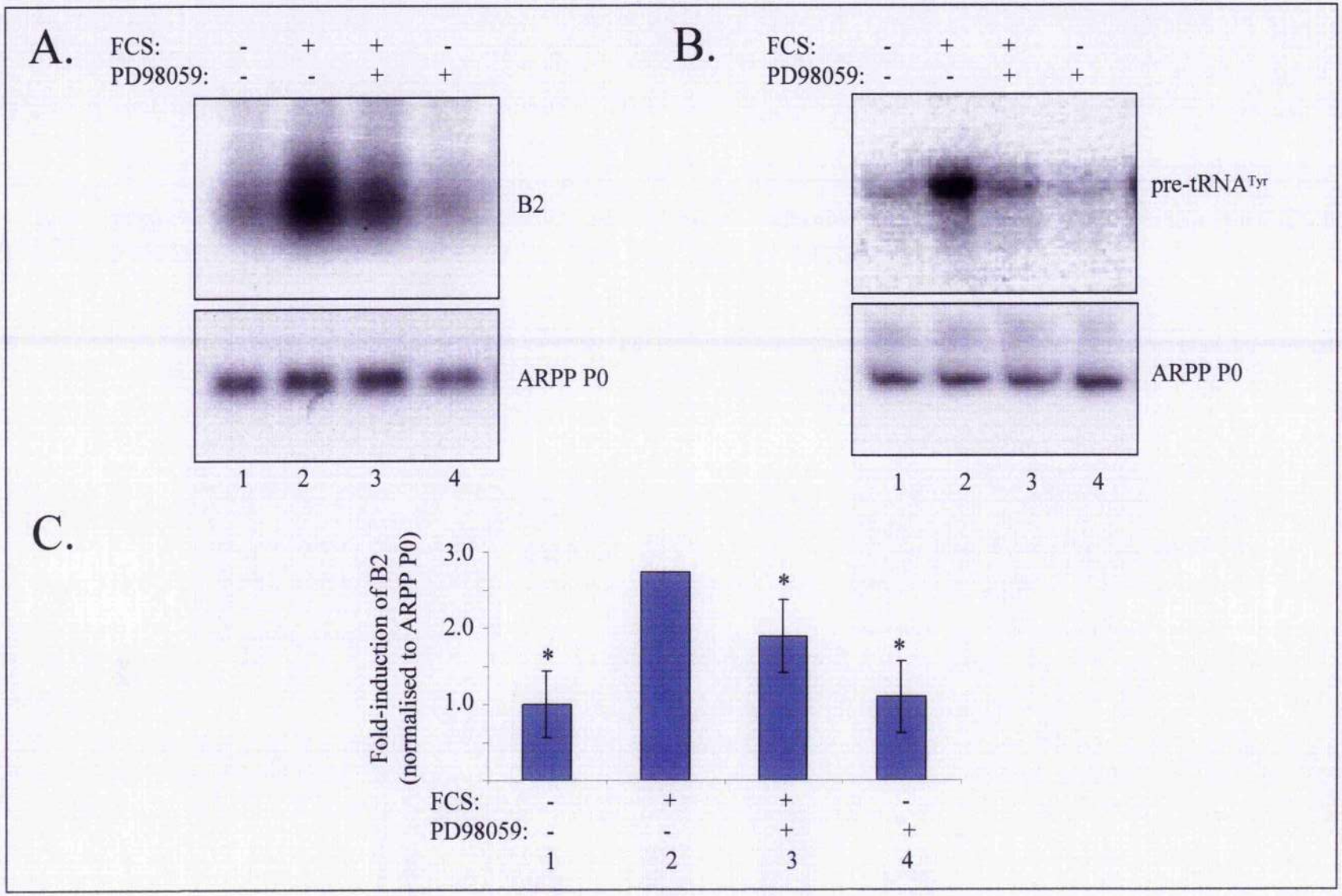

A.
FCS: - + + -
PD98059: - - + +
50kDa -
35kDa -
phos-ERK
35kDa -
total ERK
1 2 3 4
B.
protein synthesis
DNA synthesis
Fold induction of protein or DNA synthesis
3.0
2.0
1.0
0
*
**
‡
**
‡
**
FCS: - + + -
PD98059: - - + +
1 2 3 4

and 3). This suggests that ERK-independent mechanisms may also activate protein synthesis in cardiomyocytes.

To determine whether ERK is involved in the activation of pol III transcription in cardiomyocytes, the effects of both ERK inhibition (Figure 5.5) and activation (Figure 5.6) on class III gene expression were assessed. Northern blotting (Figure 5.5A) and RT-PCR analysis (Figure 5.5B) revealed that inhibiting ERK with PD98059 diminishes the increase in B2 and tRNA transcripts in response to serum. Quantification of the levels of B2 under each condition suggests that, as with protein synthesis, ERK inhibition blocks the serum-stimulated induction of pol III transcription significantly, although not completely (Figure 5.5C). Thus, evidently, ERK-independent mechanisms also contribute to the activation of pol III transcription in response to hypertrophic stimuli. These data suggest that ERK is involved in stimulating both pol III transcription and protein synthesis in cardiomyocytes, and are consistent with the hypothesis that ERK induces hypertrophic growth in a pol III-dependent manner.

Several studies have shown that ERK activation is sufficient to induce the hypertrophic growth of cardiomyocytes (Bueno et al, 2000; Gillespie-Brown et al, 1995; Ueyama et al, 2000; Wang and Proud, 2002b; Yue et al, 2000). To determine whether the activation of ERK in the absence of hypertrophic stimuli could also induce pol III transcription, cultured cardiomyocytes were infected with adenoviruses expressing constitutively active MEK1 (CAMEK) or β-galactosidase (β-gal), as a control. Other investigators have used such adenoviruses to demonstrate that ERK activation is sufficient to increase

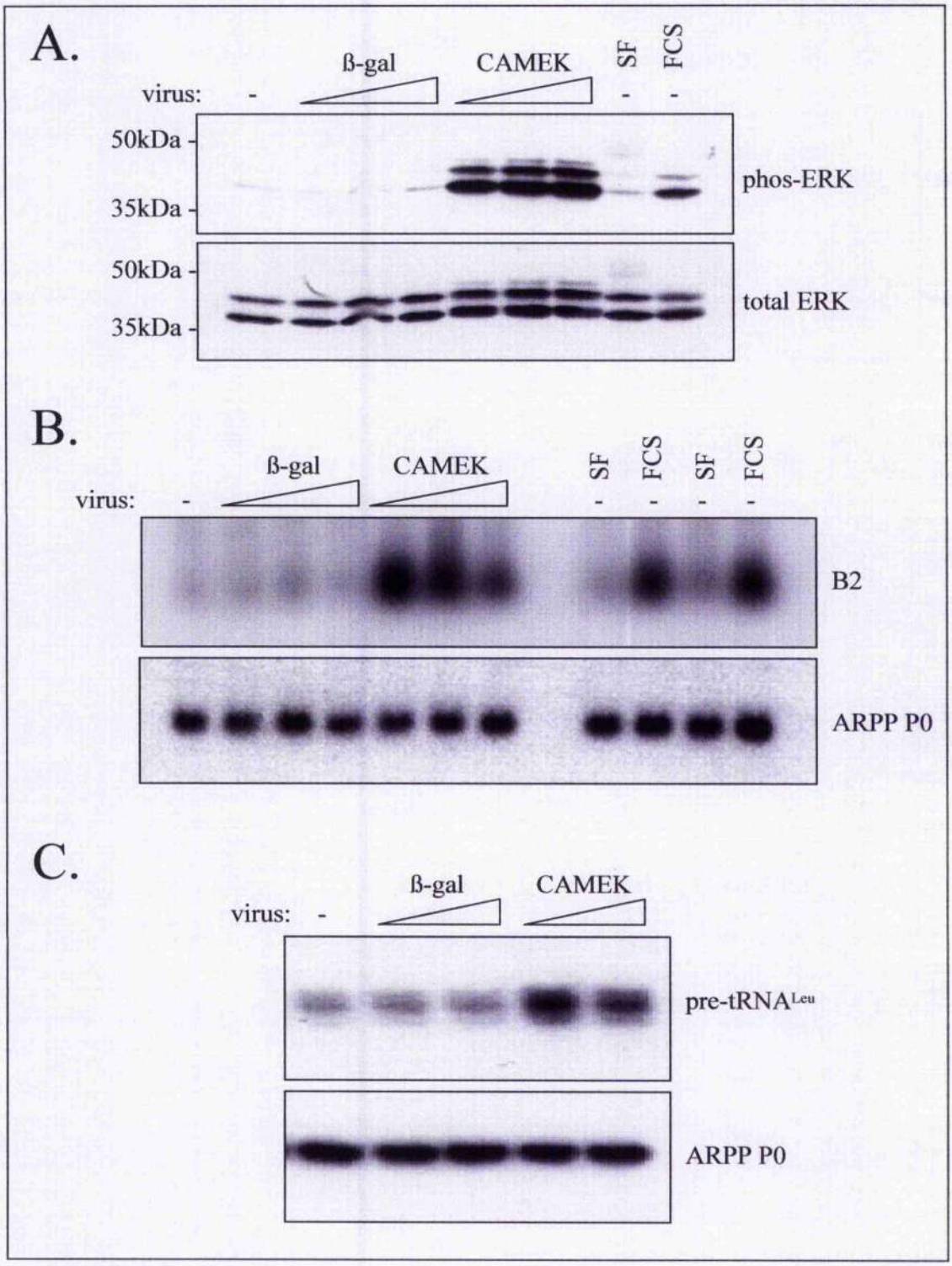
Figure 5.5: ERK inhibition impairs pol III transcription in cultured cardiomyocytes. Cardiomyocytes were treated as in Figure 5.4, then total cellular RNA extracted. A. 10 μg of RNA were analysed by Northern blotting. The upper panel shows the blot probed for B2, and the lower panel shows the same blot stripped and re-probed for ARPP P0. B. 3 μg of RNA were used to generate cDNAs by reverse transcription. Specific cDNAs were then amplified by PCR using pre-$tRNA^{Tyr}$- and ARPP P0-specific primers. C. The levels of B2 and ARPP P0 in each sample (as determined by Northern analysis) were quantified using densitometry, and B2 levels normalised against ARPP P0 levels. This was done for 4 separate experiments. The average fold increases obtained, along with standard deviations from the means, are represented in the graph (*significantly lower than condition 2, $p<0.05$).


A.
FCS: - + + -
PD98059: - - + +
B2
ARPP P0
1 2 3 4
B.
FCS: - + + -
PD98059: - - + +
pre-tRNA^{Tyr}
ARPP P0
1 2 3 4
C.
Fold-induction of B2 (normalised to ARPP P0)
3.0
2.0
1.0
*
*
*
FCS: - + + -
PD98059: - - + +
1 2 3 4

cardiomyocyte protein synthesis and size (Bueno et al, 2000; Ueyama et al, 2000; Wang and Proud, 2002b; Yue et al, 2000). Cardiomyocytes infected with varying amounts of control or CAMEK adenoviruses were cultured in serum-free conditions for 48 hours, then protein and RNA were extracted. For comparison, extracts were also prepared from uninfected cells cultured with or without 10% FCS. Western analysis confirmed that the CAMEK adenovirus effectively induces ERK phosphorylation, and hence activation (Figure 5.6A, upper panel). This is accompanied by a substantial increase in the level of B2 (Figure 5.6B) and tRNA (Figure 5.6C) transcripts, as determined by Northern blotting and RT-PCR analysis, respectively. These effects are specific, as the level of ARPP P0 mRNA is unaffected by ERK activation (Figures 5.6B and C, lower panels). Thus, ERK can specifically increase pol III transcription in cultured cardiomyocytes, in the absence of hypertrophic stimuli.

Therefore, the pol III transcription machinery is clearly a downstream target of the ERK pathway in cardiomyocytes, and this is likely to contribute to ERK-induced protein synthesis. This provides one example of a mechanism involved in the activation of pol III transcription in response to hypertrophic stimulation. However, ERK-independent mechanisms also apparently contribute to this regulation. These may involve c-Myc, RB and/or Brf1 induction, as postulated in Chapter 4. The role of c-Myc in pol III transcriptional activation is addressed in the following section.

Figure 5.6: ERK activation is sufficient to induce pol III transcription in cultured cardiomyocytes. Cultured cardiomyocytes were infected with increasing amounts (0, 10, 25 or 40 μl for A and B; 0, 25 or 40 μl for C) of adenovirus expressing CAMEK or β–gal (negative control), as indicated. Cells were serum-starved for 48 hours before extracting whole cell protein or RNA. As a positive control for ERK activation and pol III induction, uninfected cells were incubated without (SF) or with 10% FCS for 16 hours (A and B only). A. 75 μg of protein were resolved by SDS-PAGE and Western blotting was performed using the antibodies specified. B. RNA was analysed by Northern blotting. The upper panel shows the blot probed for B2, and the lower panel shows the same blot stripped and re-probed for ARPP P0. C. RNA was analysed by RT-PCR using pre-$tRNA^{Leu}$- and ARPP P0-specific primers. The viruses used for these analyses were a kind gift from Dr JD Molkentin, University of Cincinnati, USA.


A.
virus:
-
ß-gal
CAMEK
SF
FCS
50kDa
35kDa
phos-ERK
50kDa
35kDa
total ERK
B.
virus:
-
ß-gal
CAMEK
SF
FCS
SF
FCS
B2
ARPP P0
C.
virus:
-
ß-gal
CAMEK
pre-tRNA^{Leu}
ARPP P0

5.2.2 Activation of pol III transcription by c-Myc in cardiomyocytes

To determine whether c-Myc can activate pol III transcription in terminally differentiated cardiomyocytes, the effects of adenoviral-mediated c-Myc expression were investigated in culture. RNA was obtained from cardiomyocytes that had been infected (in the laboratory of Dr WR Maclellan, UCLA, USA) with adenoviruses expressing Lac Z, as a control, or c-Myc (Figure 5.7). RT-PCR analysis confirmed that the c-Myc adenovirus increased c-Myc expression in these cells, compared to cells infected with the control virus, as expected (Figure 5.7, panel D). Panels A to C (Figure 5.7) demonstrate that c-Myc overexpression is accompanied by the induction of tRNAs tyrosine, leucine and arginine. These effects are specific, as the negative control mRNA (ARPP P0) does not change in response to c-Myc expression (Figure 5.7, panel E). Therefore, these data show that c-Myc can activate class III gene expression in cultured cardiomyocytes.

In order to study the role of c-Myc in the adult myocardium, Xiao et al (2001) developed a mouse model in which an oestrogen-responsive Myc fusion protein can be activated specifically in cardiomyocytes by administering 4-hydroxytamoxifen (OHT). Transgenic mice constitutively express this c-Myc-oestrogen receptor fusion protein (MycER) in cardiomyocytes; however, in the absence of an appropriate ligand, it is sequestered in the cytoplasm and hence inactive. OHT induces MycER nuclear translocation, and thus activates c-Myc. Using this model, these investigators demonstrated that activation of c-Myc in the heart is sufficient to induce several features of cardiac hypertrophy, including an increase in cell size without cell division (Xiao et al, 2001). Hearts were obtained from these mice, and relevant control mice, and tRNA levels analysed

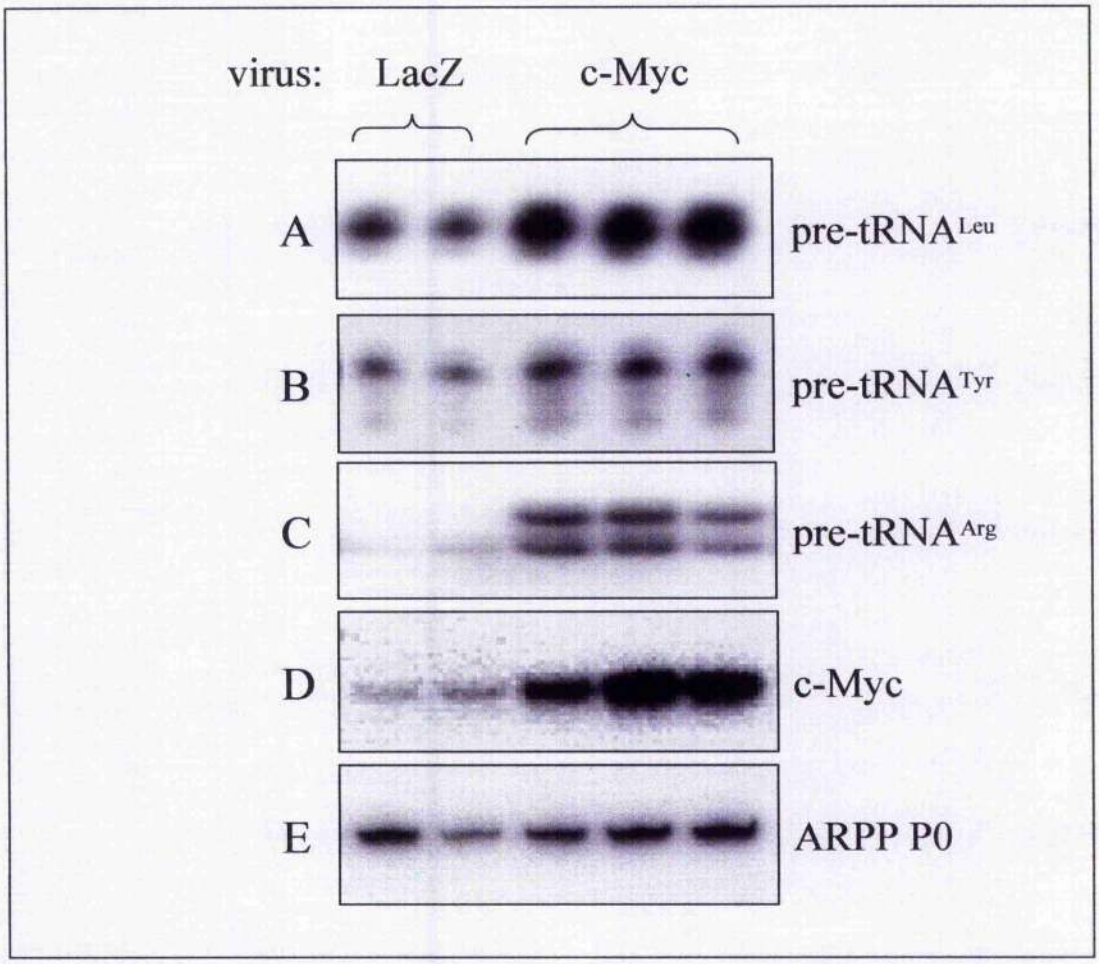


Figure 5.7: c-Myc can activate pol III transcription in cultured cardiomyocytes. Cardiomyocytes were infected with equivalent amounts of adenovirus expressing Lac Z (negative control) or c-Myc, as indicated, in the laboratory of Dr WR MacLellan (UCLA, USA). Cells were grown in the absence of hypertrophic stimuli, then total cellular RNA was extracted. This RNA was used in the current study to generate cDNAs in duplicate (Lac Z) or triplicate (c-Myc), as shown. cDNAs were amplified by PCR using primers specific for pre-tRNA^{Leu}, pre-tRNA^{Tyr}, pre-tRNA^{Arg}, c-Myc and ARPP P0, as indicated.

by RT-PCR (Figure 5.8). This revealed that c-Myc expression correlates with a substantial increase in the levels of pre-tRNA^{Tyr}, pre-tRNA^{Leu} and pre-tRNA^{Arg}, indicating that c-Myc activates pol III transcription in the heart (Figure 5.8, panels A to C). This is the first demonstration that c-Myc can induce pol III transcription *in situ*. As a positive control, the level of cyclin D2 mRNA was also measured (Figure 5.8, panel D). Cyclin D2 is a well-characterised direct target of c-Myc, and is known to respond to c-Myc in proliferating cells and cardiomyocytes (Bouchard et al, 1999; Bouchard et al, 2001; Coller et al, 2000; Xiao et al, 2001). As expected, c-Myc increases the expression of this gene. However, the induction of pol III transcripts by c-Myc appears to be stronger than the induction of cyclin D2. This observation is similar to effects described by Gomez-Roman et al (2003): in this previous study, c-Myc caused a much more robust activation of tRNA expression than cyclin D2 expression in fibroblasts.

In proliferating cells, c-Myc associates with class III genes, and this is assumed to explain the activation of pol III transcription by this transcription factor (Felton-Edkins et al, 2003b; Gomez-Roman et al, 2003). To test whether c-Myc was associated with class III gene promoters in cardiomyocytes *in vivo*, cultured cells were grown under hypertrophic conditions, then ChIP assays carried out using an anti-c-Myc antibody (Figure 5.9A). As a positive control, an antibody against the TFIIIB component TBP was employed, and as negative controls, a TFIIB antibody and beads alone were used. As expected, TBP associates with promoters utilised by pol III in cardiomyocytes, including tRNA^{Leu}, as shown in Figure 5.9A (lane 1). However, no binding of c-Myc to tRNA^{Leu}, tRNA^{Tyr} nor 5S

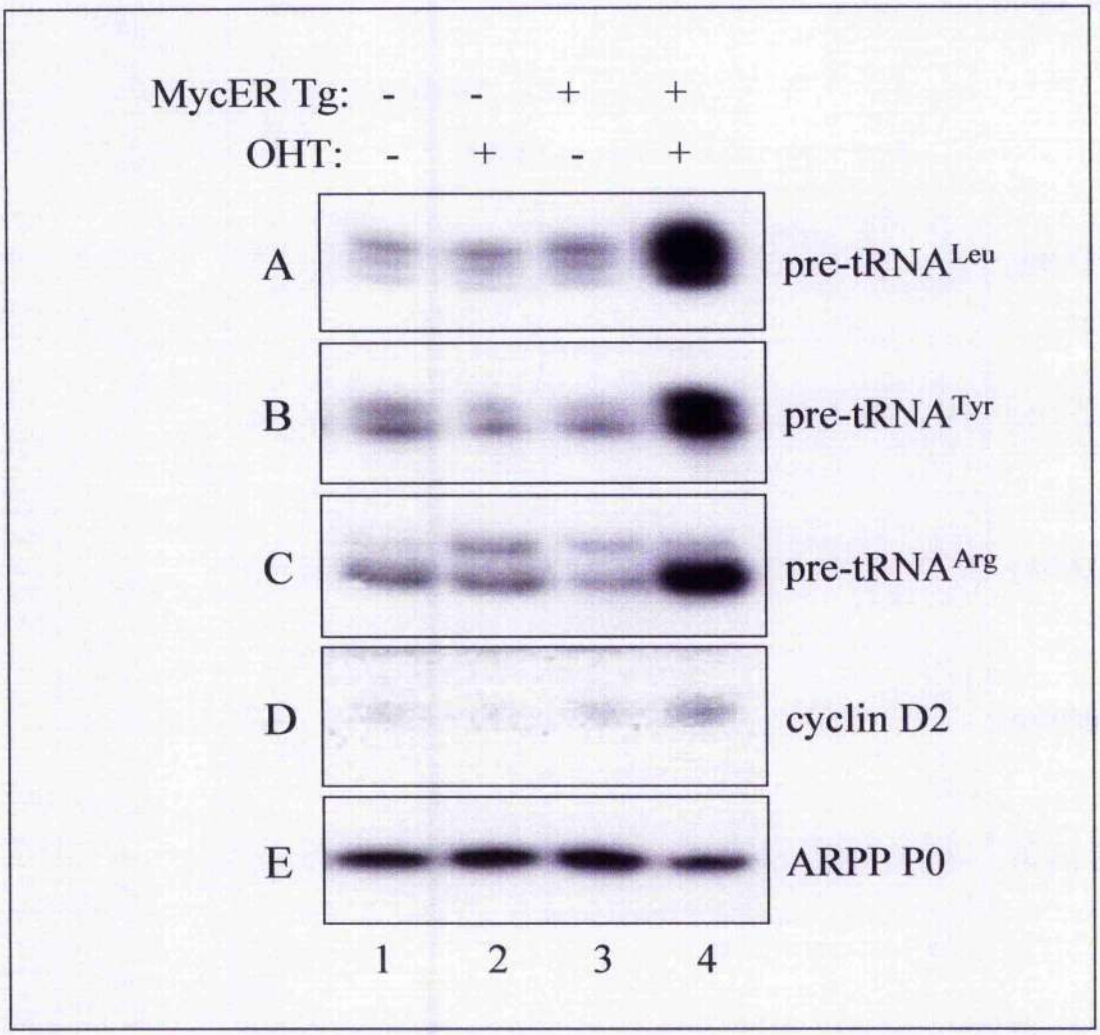
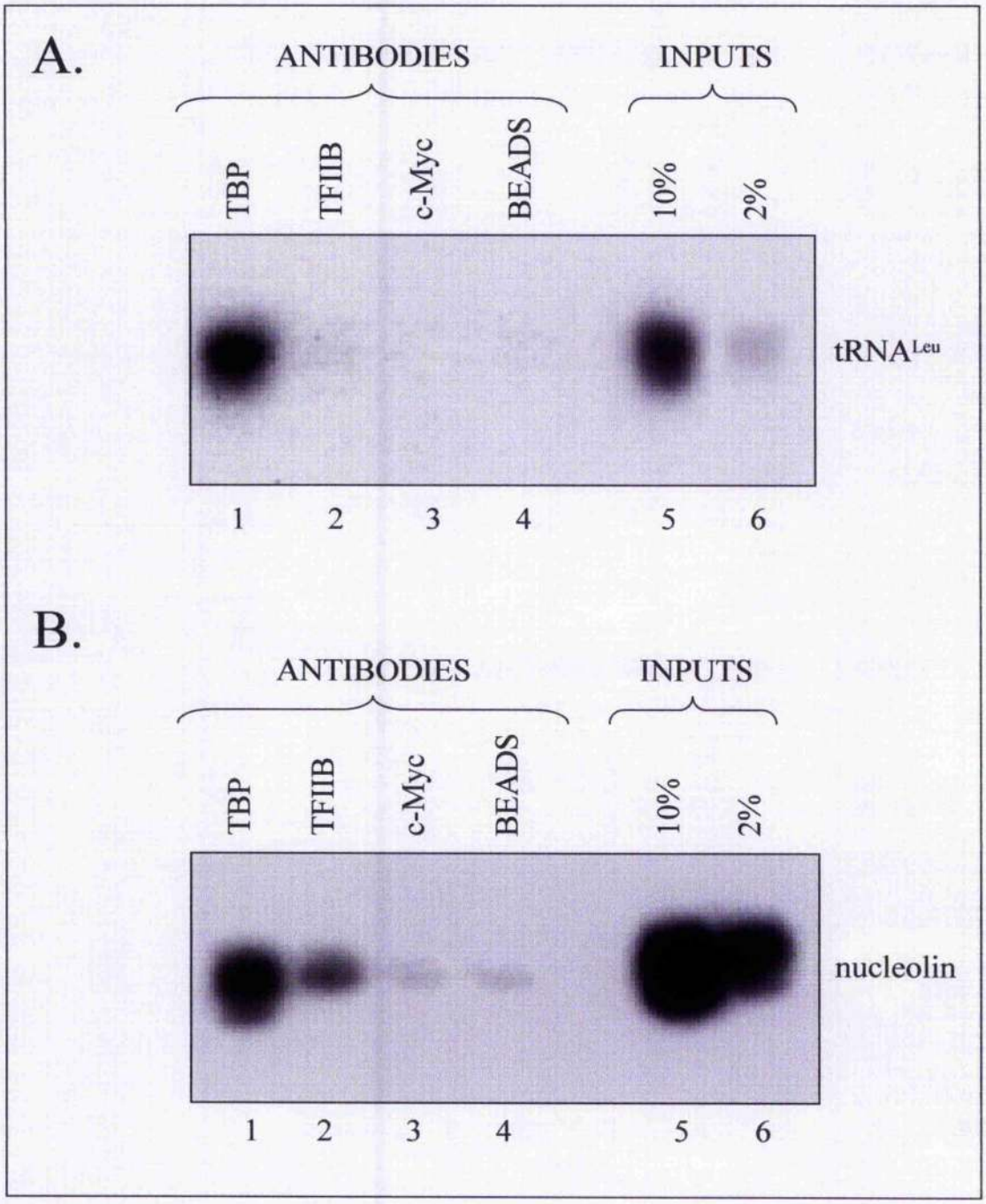


Figure 5.8: c-Myc can activate pol III transcription in the heart. RNA was extracted from hearts derived from wild-type adult mice (lanes 1 and 2) or transgenic littermates expressing a MycER fusion protein specifically in cardiomyocytes (MycER Tg, lanes 3 and 4). Mice had either been exposed to OHT (lanes 2 and 4) or vehicle (peanut oil, lanes 1 and 3) for 1 week prior to removal of hearts. RNA was analysed by RT-PCR using pre-tRNA^{Leu}-, pre-tRNA^{Tyr}-, pre-tRNA^{Arg}-, cyclin D2- and ARPP P0-specific primers, as specified. The hearts used for these analyses were obtained from Dr WR MacLellan, UCLA, USA.

Figure 5.9: Analysis of c-Myc binding to promoters in cardiomyocytes *in vivo*. Cultured cardiomyocytes were grown in the presence of 10% FCS to induce hypertrophic growth. ChIPs were performed with antibodies against TBP, TFIIB, c-Myc or with beads alone. A. c-Myc binding to class III gene promoters cannot be detected in cultured cardiomyocytes. The association of each factor with the $tRNA^{Leu}$ promoter is shown. Similar results were obtained for the $tRNA^{Tyr}$ and 5S rRNA genes (data not shown), as determined by PCR with gene-specific primers. B. c-Myc binding to the nucleolin promoter cannot be detected in cultured cardiomyocytes, as determined by PCR with primers designed to amplify the nucleolin E-box. Input genomic DNA (10% and 2% of that used in the ChIPs) was analysed in the same PCR reactions (A and B, lanes 5 and 6). In the examples shown, the c-Myc antibody 9E10 was used. However, the data are also representative of results obtained using the N262 c-Myc antibody, which recognises a distinct epitope on the c-Myc protein.


A.
ANTIBODIES
INPUTS
TBP
TFIIB
c-Myc
BEADS
10%
2%
tRNA^Leu
1
2
3
4
5
6
B.
ANTIBODIES
INPUTS
TBP
TFIIB
c-Myc
BEADS
10%
2%
nucleolin
1
2
3
4
5
6

rRNA promoters could be detected in these cells. Similar results were obtained with two distinct c-Myc antibodies. Absence of c-Myc association with $tRNA^{Leu}$ promoters is shown in Figure 5.9A (lane 3) as an example. This may indicate that c-Myc does not interact with class III gene promoters in cardiomyocytes, thus inferring activation of pol III transcription by more indirect mechanisms. Alternatively, c-Myc may not have been effectively immunoprecipitated from these cells.

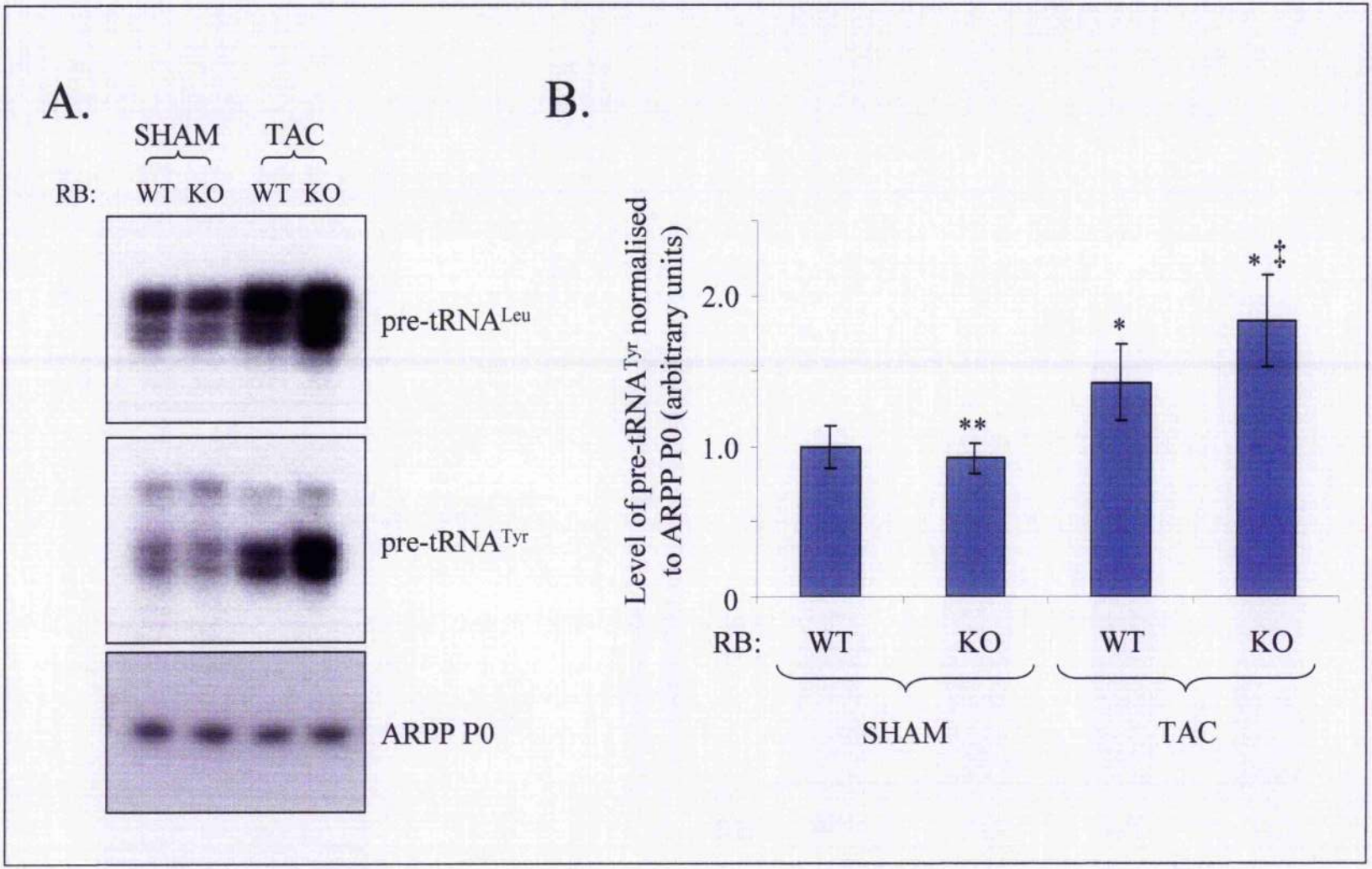
To test whether lack of c-Myc association was specific to pol III promoters, or likely to be as a result of inadequate c-Myc immunoprecipitation, the binding of c-Myc to another known target gene, nucleolin, was investigated (Coller et al, 2000; Greasley et al, 2000; O'Connell et al, 2003). Unlike class III genes, the pol II-transcribed nucleolin gene contains the characteristic consensus E-box binding site for c-Myc (Frank et al, 2001; Greasley at al, 2000). Previous studies have demonstrated, by ChIP analyses, that c-Myc binds the nucleolin promoter in rat cells *in vivo* (Frank et al, 2001; Zeller et al, 2001). Furthermore, c-Myc induction activates nucleolin expression in cardiomyocytes (Xiao et al, 2001). Figure 5.9B shows that both TBP and TFIIB bind to the nucleolin promoter (lanes 1 and 2), as expected since both of these factors are required for pol II-dependent transcription. In contrast, c-Myc binding to this promoter was not significantly above background levels (Figure 5.9B, compare lanes 3 and 4). This is consistent with a failure to immunoprecipitate c-Myc in this assay. Therefore, further work is required to determine the mechanisms by which c-Myc activates pol III transcription in cardiomyocytes.

Nonetheless, the data clearly suggest that c-Myc activates the pol III-dependent transcription of essential components of the cellular biosynthetic machinery, both in cultured cardiomyocytes and in the heart. Potentially, this may contribute to the induction of hypertrophic growth by c-Myc.

5.2.3 Regulation of pol III transcription by RB in cardiomyocytes

To establish whether RB is necessary for restraining pol III activity in the heart, the effects of RB deletion were analysed. Dr WR MacLellan and colleagues (UCLA, USA) have created transgenic mice in which RB has been effectively deleted specifically from cardiomyocytes. These mice develop normally, and basal heart function is unaffected (MacLellan et al, unpublished results). However, in response to hypertrophic stimuli, RB-null mice display significantly exaggerated heart growth, compared to RB-expressing littermates. RNA was extracted from hearts derived from these mice, and analysed by RT-PCR (Figure 5.10). This revealed a striking correlation between the level of pol III transcripts and heart growth. Thus, in the absence of hypertrophic stimulation (sham-operated hearts), tRNA levels are unaffected by RB deletion, as is heart size and function (MacLellan et al, unpublished results). However, the level of tRNA in hearts subjected to TAC is on average 30% higher in hearts lacking RB, than in wild-type control hearts. This effect is specific, as indicated by the constant level of ARPP P0 in each heart. Similarly, the average TAC-induced increase in heart weight (normalised to body weight) was significantly enhanced (by approximately 25%) in the absence of RB (MacLellan et al, unpublished results). Therefore, loss of RB augments the increase in pol III transcription and

Figure 5.10: Loss of RB augments the activation of pol III transcription in response to hypertrophic stimulation of the heart. Hearts were obtained from adult mice expressing wild-type RB (WT) or sibling mice lacking RB (KO) specifically in cardiomyocytes. Prior to heart removal, pressure-overload hypertrophy was induced by TAC for 2 weeks. Control animals underwent sham operation (SHAM). RNA was extracted from whole hearts, then used for RT-PCR analysis with pre-tRNA^{Leu}-, pre-tRNA^{Tyr}- and ARPP P0-specific primers, as indicated. A. RT-PCRs, using RNA derived from 1 animal per condition, are shown. B. Densitometry was used to quantify the amounts of pre-tRNA^{Tyr} and ARPP P0 in each heart. Four hearts were analysed for each condition, and the average level of pre-tRNA^{Tyr}, normalised to ARPP P0, is shown in the graph. The error bars indicate the standard deviation from the mean (*significantly higher than WT SHAM, $p<0.05$; ‡ significantly higher than WT TAC, $p<0.05$; **not significantly different from WT SHAM). The hearts used for these analyses were provided by Dr WR MacLellan, UCLA, USA.


A.
SHAM
TAC
RB:
WT KO WT KO
pre-tRNA^Leu
pre-tRNA^Tyr
ARPP P0
B.
Level of pre-tRNA^Tyr normalised to ARPP P0 (arbitrary units)
2.0
1.0
0
**
*
* ‡
RB:
WT
KO
WT
KO
SHAM
TAC

hypertrophic growth in response to pressure-overload. This is consistent with a role for RB in restraining class III gene expression, and thus hypertrophy, in the heart under certain conditions.

Having determined that RB can repress pol III transcription in cardiomyocytes, the next aim was to investigate whether RB phosphorylation contributes to the derepression of pol III transcription during hypertrophy. Cyclin induction and RB phosphorylation characteristically accompany the serum stimulation of proliferating cells (Hulleman and Boonstra, 2001). As discussed in Chapter 3, a small pool of such cells is likely to be present in the primary cultures used here. Therefore, before examining the role of RB phosphorylation in the upregulation of pol III transcription in cultured cardiomyocytes, it seemed pertinent to exclude the possibility that the observed increases in cyclin D1 and RB phosphorylation result from a proliferative, rather than a hypertrophic, response. To address this issue, cardiomyocytes were cultured in the presence or absence of BrdU, and the effects of serum stimulation on cyclin D1 levels and RB phosphorylation status were compared by Western blotting. As shown in Figure 5.11, BrdU has little effect on the induction of cyclin D1 or RB phosphorylation by FCS. This confirms that these effects do indeed accompany cardiomyocyte hypertrophy.

CDKs are positively regulated by the appropriate cyclin partners. However, CDK activity is also negatively regulated by various CDK inhibitor (CKIs) proteins, belonging to the CIP/KIP and INK4 families (reviewed in Sherr and Roberts, 1999). Various CKIs are expressed in the heart, including $p21^{CIP1}$ and $p27^{KIP1}$, which belong to the CIP/KIP family (Brooks et al, 1998; Koh et al, 1998;

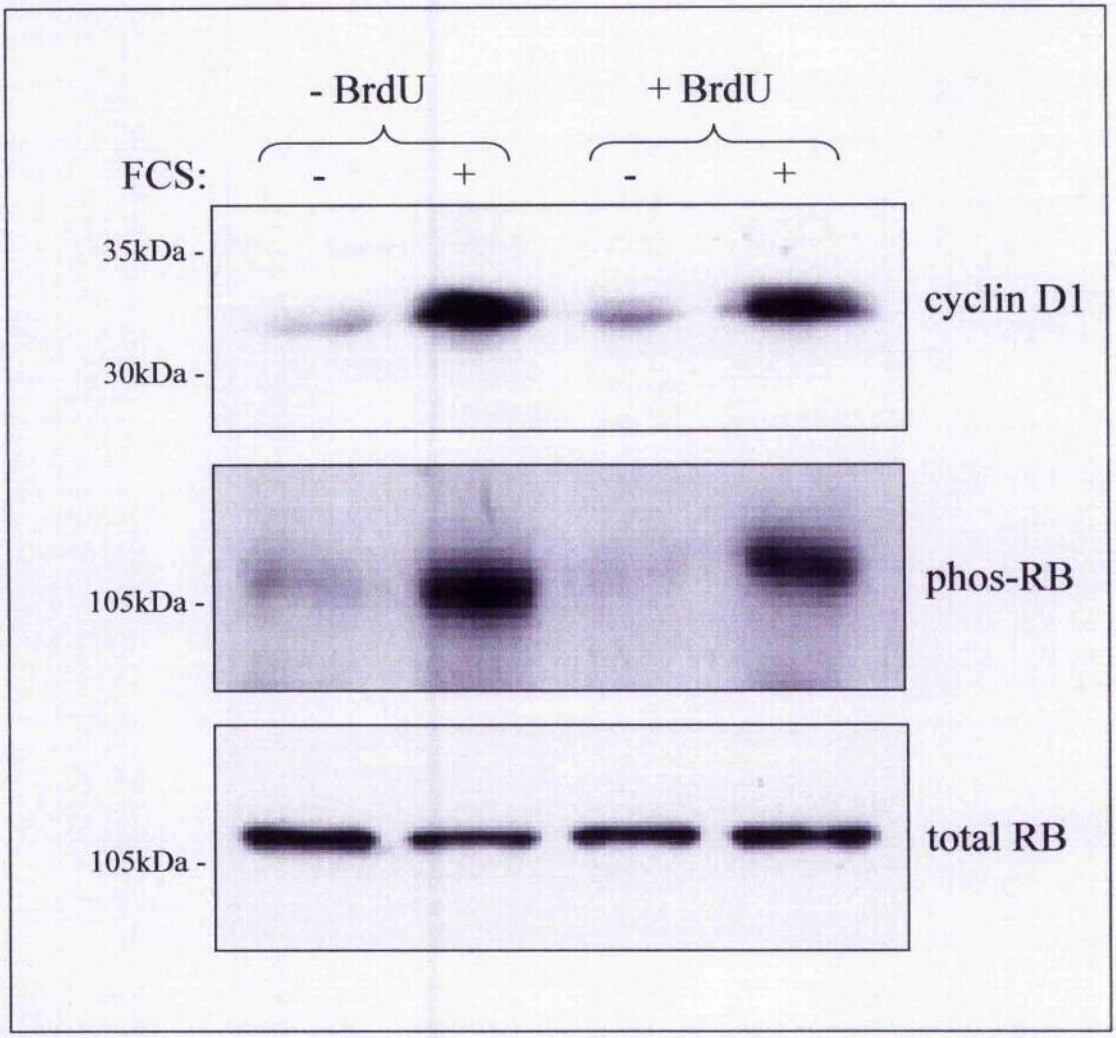
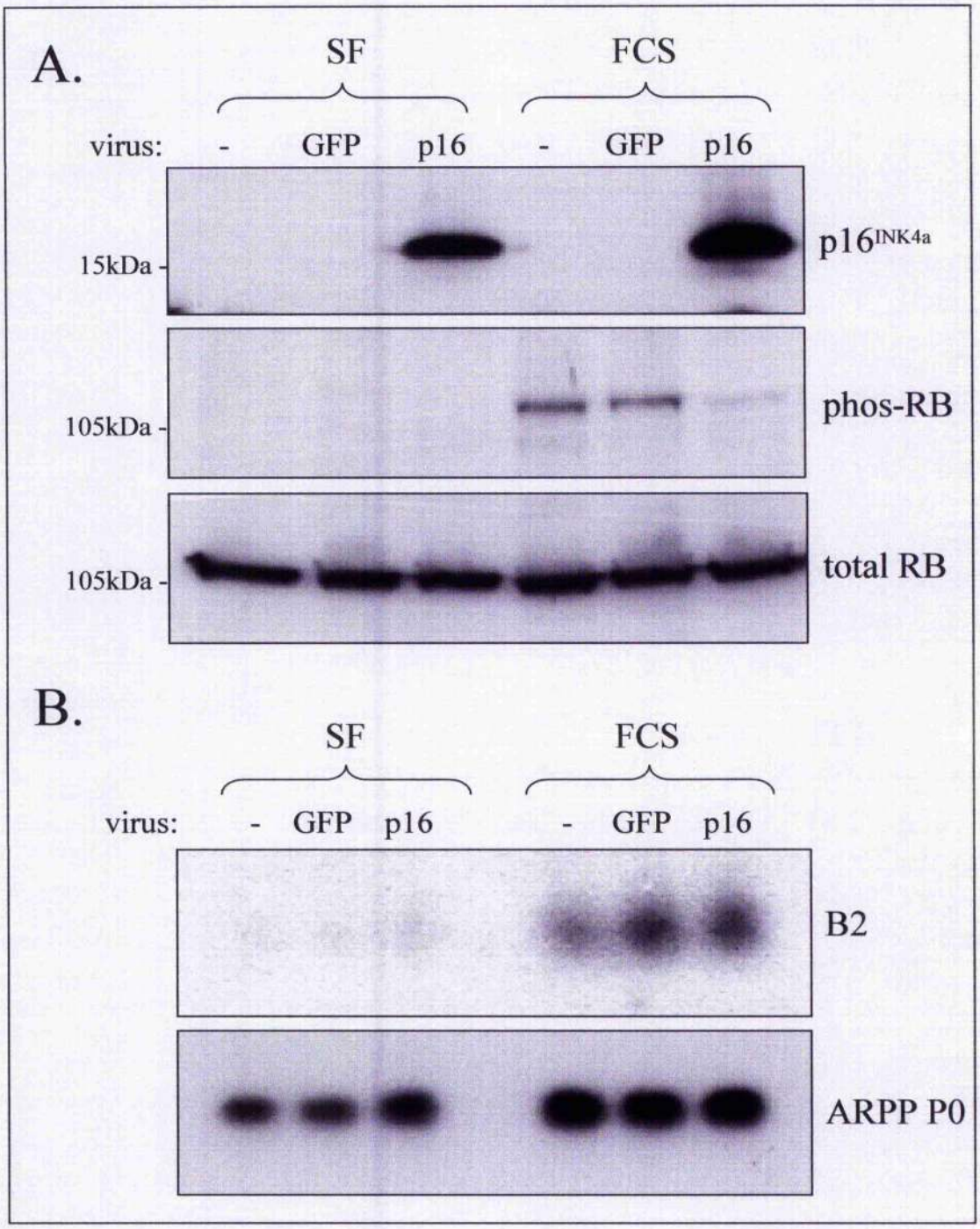


Figure 5.11: RB phosphorylation in cultured cardiomyocytes is not due to proliferating cells. Following dissociation from neonatal rat hearts, cardiomyocytes were cultured in media containing 0.1 mM BrdU or vehicle alone (0.1% DMSO). BrdU and vehicle exposed cells were serum starved for 24 hours, then treated with or without 10% FCS for 16 hours to induce hypertrophy. BrdU or vehicle were present throughout the course of the experiment. Whole cell lysates were prepared and 75 μg of protein analysed by Western blotting using the antibodies indicated. The phos-RB antibody used specifically detects RB phosphorylated on thr 826.

Poolman and Brooks, 1998). These proteins have been implicated in the development and maintenance of cardiomyocyte terminal differentiation (Brooks et al, 1998; Flink et al, 1998; Koh et al, 1998; Poolman and Brooks, 1998; Poolman et al, 1999). In contrast, members of the INK4 family, including $p16^{INK4a}$, do not appear to be expressed in differentiated cardiomyocytes (Brooks et al, 1998; Koh et al, 1998; Nozato et al, 2001). INK4 CKIs specifically block cyclin D-dependent kinases, and thereby directly prevent RB phosphorylation on CDK4/6-specific sites. In proliferating cells, $p16^{INK4a}$ expression is sufficient to induce cell cycle arrest, indicating that phosphorylation by $p16^{INK4a}$-inhibited kinases is essential for the inactivation of RB in these cells (Medema et al, 1995). Accordingly, ribozyme-mediated depletion of $p16^{INK4a}$ in fibroblasts activates pol III transcription (Scott et al, 2001). To determine if RB phosphorylation is required for the activation of pol III transcription in cardiomyocytes, $p16^{INK4a}$ was overexpressed in cultured cells using adenoviral-mediated gene delivery. Western blotting confirmed that $p16^{INK4a}$ was expressed in cells infected with the $p16^{INK4a}$ adenovirus, but not in those infected with a GFP-expressing control virus (Figure 5.12A, upper panel). Furthermore, $p16^{INK4a}$ expression effectively blocked the phosphorylation of RB in response to FCS, without affecting the total level of RB (Figure 5.12A, lower two panels). To determine whether this influenced the induction of pol III transcription by FCS, Northern blotting was performed. Figure 5.12B demonstrates that the increase in B2 transcripts in response to serum was unaffected by $p16^{INK4a}$ expression. This indicates that the activation of pol III transcription by FCS does not require RB phosphorylation in cultured cardiomyocytes, thus implying that active RB is not sufficient to block the induction of pol III transcription in response to hypertrophic stimulation.

Figure 5.12: $p16^{INK4a}$ inhibits RB phosphorylation, but not pol III transcription, in cultured cardiomyocytes. Cultured cardiomyocytes were infected with or without (-) equivalent amounts of adenovirus expressing GFP (negative control) or $p16^{INK4a}$ (p16), as indicated. Cells were serum starved (SF) or stimulated with 10% FCS for 16 hours prior to extracting whole cell protein or RNA. A. Proteins were resolved by SDS-PAGE and Western blotting was performed using antibodies against the proteins specified. The phos-RB antibody used specifically detects RB phosphorylated on thr 826. B. RNA was analysed by Northern blotting. The upper panel shows the blot probed for B2, and the lower panel shows the same blot stripped and re-probed for ARPP P0. The viruses used for these analyses were a kind gift from Dr S Mittnacht, Institute of Cancer Research, UK.


A.
SF
FCS
virus:
-
GFP
p16
-
GFP
p16
15kDa
p16INK4a
105kDa
phos-RB
105kDa
total RB
B.
SF
FCS
virus:
-
GFP
p16
-
GFP
p16
B2
ARPP P0

5.2.4 Regulation of c-Myc and RB via ERK in cardiomyocytes

The data presented above suggest that ERK and c-Myc can activate pol III transcription in cardiomyocytes, whereas RB may be required to restrict this activation. ERK is a central regulator of cellular function, and is known to participate in the control of c-Myc and RB in various cell types (Cheng et al, 1999; Garrington and Johnson, 1999; Hulleman and Boonstra, 2001; Sears et al, 2000; Wilkinson and Millar, 2000). This raises the possibility that ERK not only induces pol III transcription directly, by phosphorylating TFIIIB, as occurs in proliferating cells (Felton-Edkins et al, 2003a), but also indirectly, by influencing the activity of c-Myc and/or RB. Therefore, the regulation of these proteins by ERK was investigated.

To determine whether c-Myc is induced by ERK in cultured cardiomyocytes, the CAMEK adenovirus was used to specifically activate ERK in the absence of hypertrophic stimuli. Western blotting of extracts derived from cells infected with CAMEK or control virus revealed that c-Myc was indeed induced at the protein level in response to ERK activation (Figure 5.13). Thus, this may contribute to ERK-induced pol III transcription.

To establish whether ERK signalling is necessary for the induction of c-Myc during hypertrophy, ERK activation was inhibited using PD98059. Although this inhibitor clearly blocks the phosphorylation of ERK, it does not influence the induction of c-Myc by FCS (Figure 5.14). This suggests that ERK-independent pathways are sufficient to induce c-Myc in cardiomyocytes. The induction of c-Myc by FCS in the presence of PD98059 may account for the previously

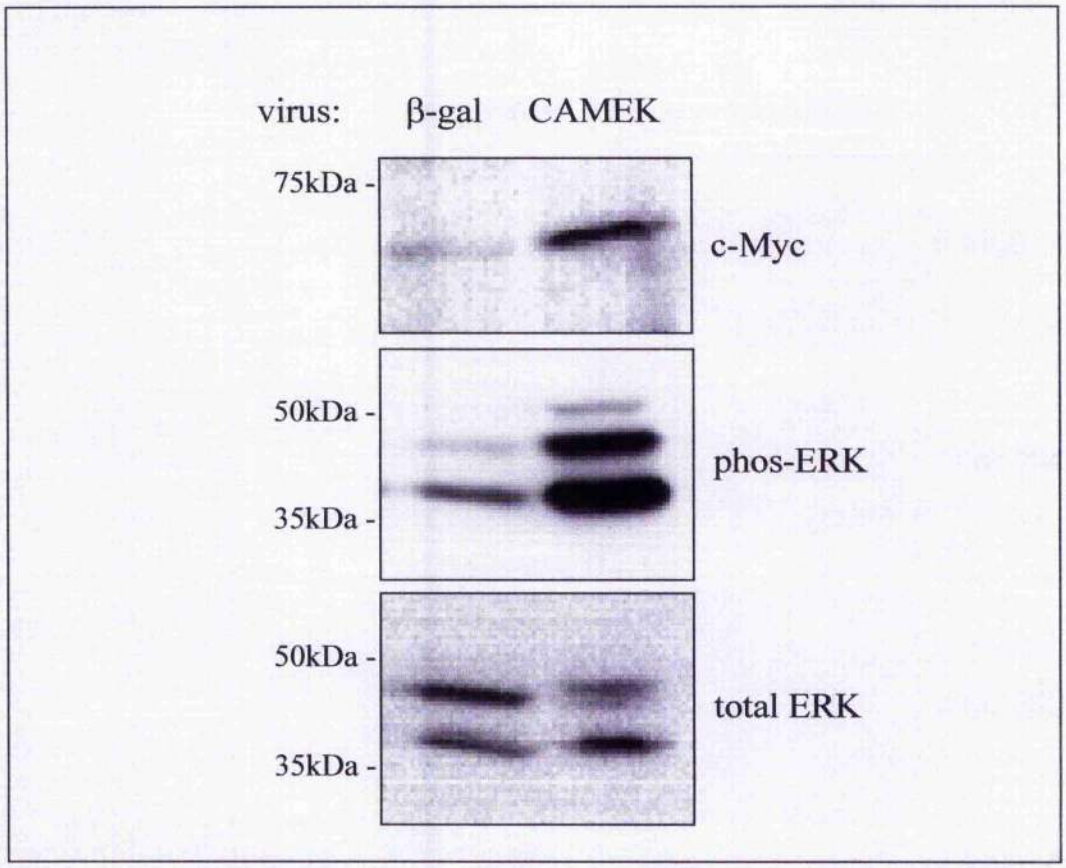


Figure 5.13: ERK activation is sufficient to induce c-Myc in cultured cardiomyocytes. Cultured cardiomyocytes were infected with equivalent amounts (25 μl) of adenovirus expressing CAMEK or β–gal (negative control), as indicated. Cells were serum starved for 48 hours prior to extracting whole cell protein. 75 μg of protein were resolved by SDS-PAGE and Western blotting was performed using antibodies against the proteins specified. The viruses used for these analyses were a kind gift from Dr JD Molkentin, University of Cincinnati, USA.

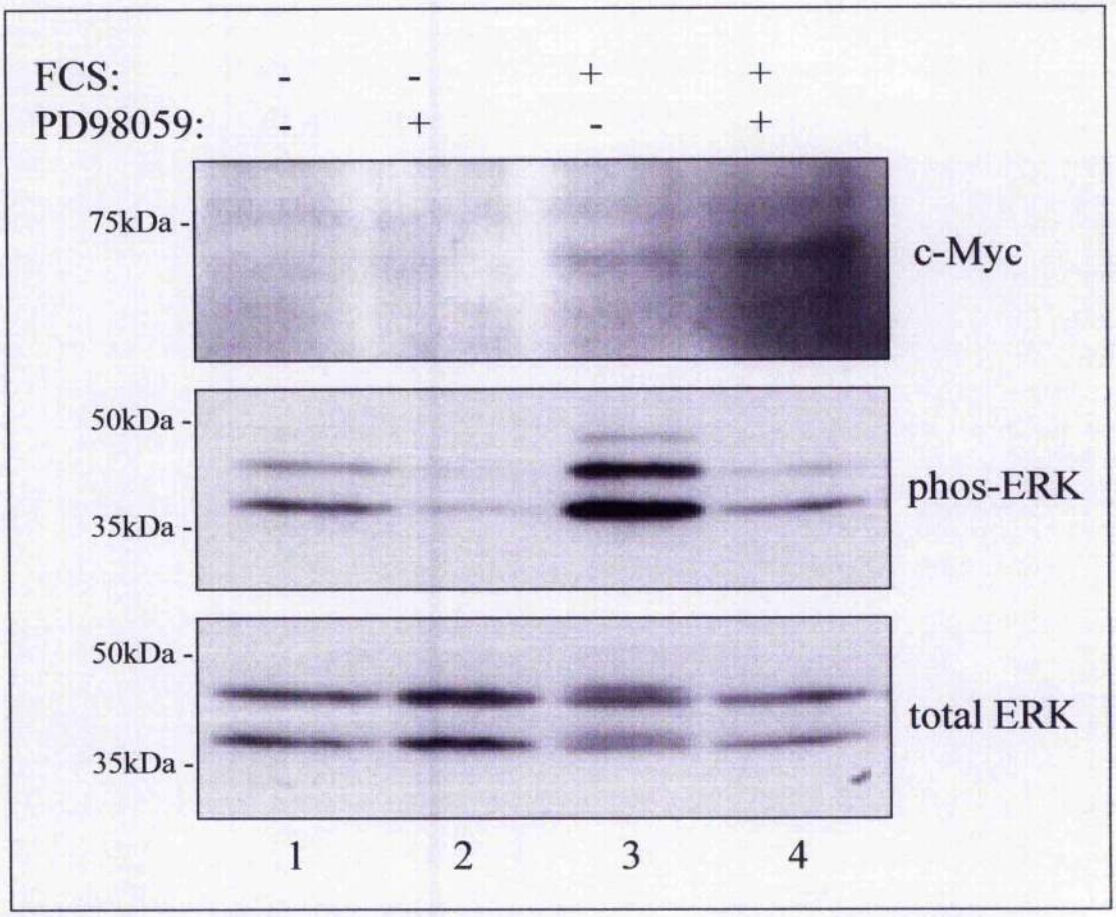


Figure 5.14: ERK inhibition does not affect c-Myc levels. Cardiomyocytes were cultured in the absence of serum for 24 hours, then either maintained in serum-free media for a further 16 hours (conditions 1 and 2), or stimulated with 10% FCS for 16 hours (conditions 3 and 4). 50 μM PD98059 (conditions 2 and 4) or vehicle alone (0.1% DMSO; conditions 1 and 3) were also included, as indicated. Whole cell lysates were prepared and 75 μg of protein resolved by SDS-PAGE. Western blotting was performed with antibodies against the proteins specified.

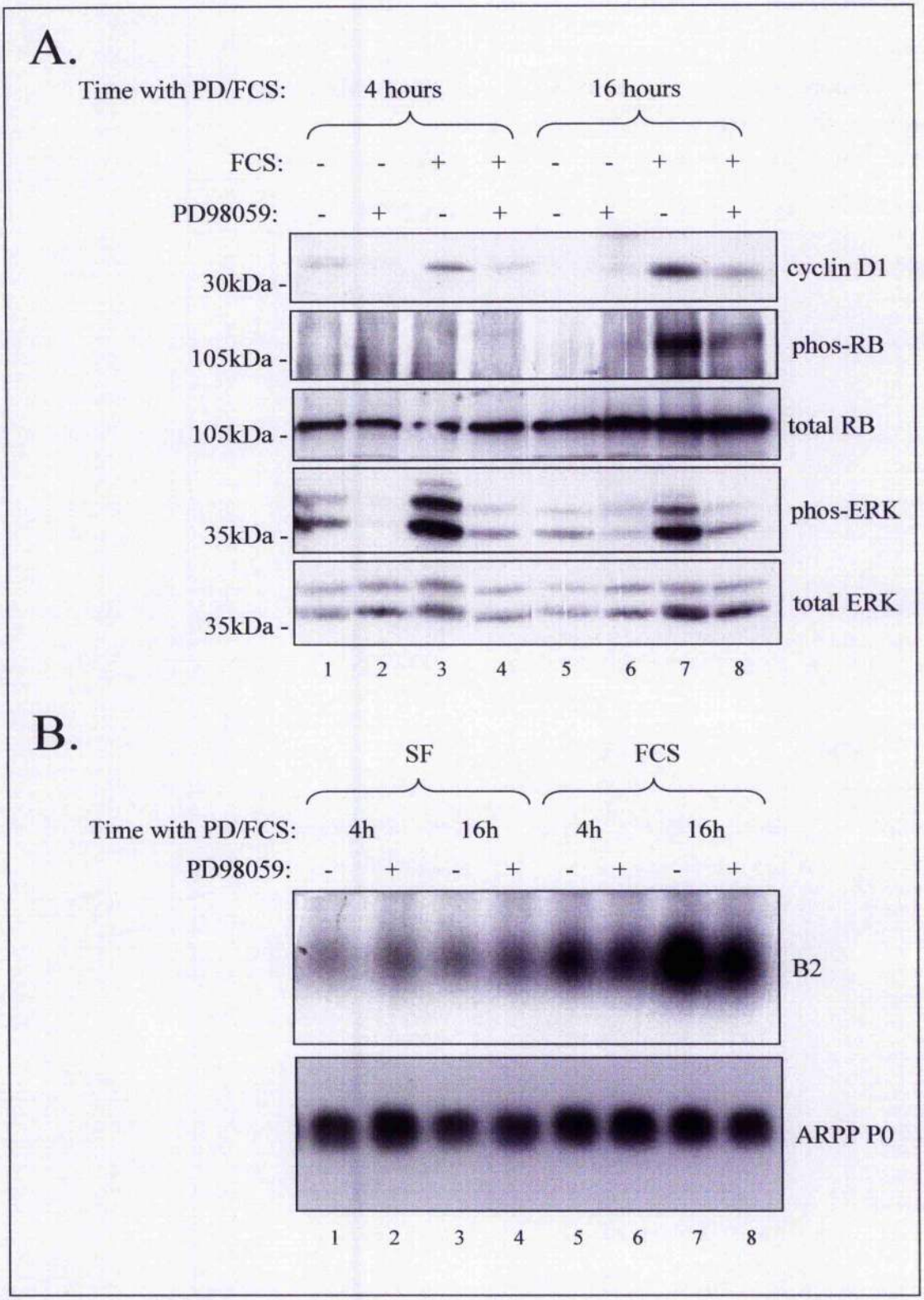
described partial activation of pol III transcription in the presence of this inhibitor (Figure 5.5). However, ERK activation is clearly necessary for the full induction of pol III transcription.

Thus, although ERK may cause c-Myc induction under certain circumstances, which would likely contribute to its activation of pol III transcription, ERK also appears to induce pol III transcription independently of c-Myc, potentially by directly phosphorylating TFIIIB.

The Raf/MEK/ERK pathway also contributes to growth factor-dependent cyclin D induction, and thus RB phosphorylation, in proliferating cells (Hulleman and Boonstra, 2000; Lavoie et al, 1996; Weber et al, 1997; Wilkinson and Millar, 2000). ERK-dependent RB phosphorylation was examined in cultured cardiomyocytes in the present study. ERK inhibition blocks both cyclin D1 induction and RB phosphorylation following 16 hours of serum stimulation, suggesting that the ERK pathway is involved in the regulation of these proteins in cardiomyocytes (Figure 5.15A, compare lanes 7 and 8). Thus, ERK-induced RB phosphorylation may contribute to the increase in pol III transcripts after 16 hours of serum-stimulation.

However, as described previously, blocking RB phosphorylation with $p16^{INK4a}$ has no effect on the level of pol III transcripts induced by FCS (Figure 5.12). Therefore, it is unlikely that ERK inhibition impairs pol III transcription solely by preventing RB phosphorylation.

Figure 5.15: Effects of ERK inhibition on cyclin D1 expression, RB phosphorylation and pol III transcription in cultured cardiomyocytes. Cardiomyocytes were cultured in the absence of serum for 24 hours, then either maintained in serum-free (SF) media for a further 4 or 16 hours, or stimulated with 10% FCS for 4 or 16 hours, as indicated. 50 μM PD98059 (PD) or vehicle alone (0.1% DMSO) were also included for 4 or 16 hours, as specified. A. Whole cell lysates were analysed by Western blotting using the indicated antibodies. The phos-RB antibody used specifically detects RB phosphorylated on thr 826. B. Whole cell RNA was analysed by Northern blotting. The upper and lower panels show the blot probed for B2 then re-probed for ARPP P0, respectively. The figures shown are representative of three independent experiments.


A.
Time with PD/FCS:
4 hours
16 hours
FCS: - - + + - - + +
PD98059: - + - + - + - +
30kDa -
cyclin D1
105kDa -
phos-RB
105kDa -
total RB
35kDa -
phos-ERK
35kDa -
total ERK
1 2 3 4 5 6 7 8
B.
SF
FCS
Time with PD/FCS:
4h
16h
4h
16h
PD98059: - + - + - + - +
B2
ARPP P0
1 2 3 4 5 6 7 8

ERK is phosphorylated within 1 hour of hypertrophic stimulation, whereas RB phosphorylation is not apparent until after 12 hours (Figure 4.6). To confirm that ERK is required for the activation of pol III transcription, independently of its regulation of RB, the effects of PD98059 on blocking pol III transcription were also examined prior to RB phosphorylation (Figure 5.15B, compare lanes 5 and 6). This revealed that ERK is involved in the early activation of pol III transcription during hypertrophic growth. Furthermore, Figure 5.16 demonstrates that ERK activation (by CAMEK expression), which is sufficient to induce pol III transcription in cardiomyocytes (Figure 5.6), is not sufficient to induce RB phosphorylation, despite increasing cyclin D1 levels. Thus, together, these data suggest that ERK activates pol III transcription independently of RB phosphorylation, and are consistent with the effects of $p16^{INK4a}$ overexpression (Figure 5.12), which indicate that cyclin D-dependent RB phosphorylation is not essential for the activation of pol III transcription by hypertrophic stimuli. However, these experiments do not discount the possibility that RB limits the extent to which pol III transcription is activated during hypertrophic growth.

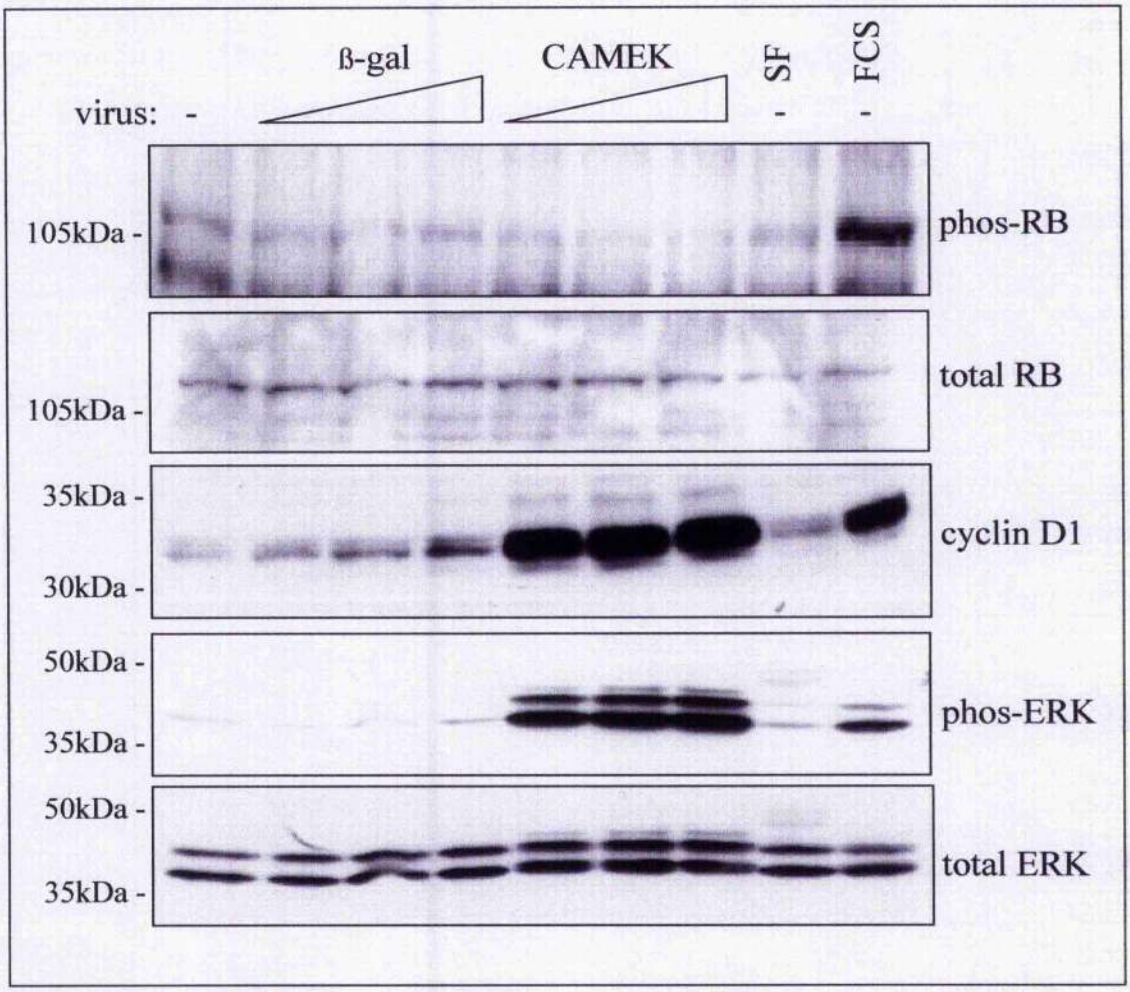


Figure 5.16: ERK activation does not induce RB phosphorylation in cultured cardiomyocytes. Cultured cardiomyocytes were infected with increasing amounts of adenovirus expressing CAMEK or β–gal (negative control), as indicated. Cells were serum starved for 48 hours before extracting whole cell protein. As a positive control for ERK activation, cyclin induction and RB phosphorylation, uninfected cells were stimulated with or without (SF) 10% FCS for 16 hours. Proteins were resolved by SDS-PAGE and Western blotting was performed using the antibodies specified. The phos-RB antibody used specifically detects RB phosphorylated on thr 826. The viruses used for these analyses were a kind gift from Dr JD Molkentin, University of Cincinnati, USA.

5.3 Discussion

Chapter 4 implicated ERK, c-Myc and RB in the regulation of pol III transcription in cardiomyocytes. The data presented in this chapter confirm that, in these cells, class III gene expression is induced by ERK and c-Myc, but repressed by RB, at least under certain circumstances. There is a clear correlation between this regulation of pol III transcription and the involvement of each of these proteins in the hypertrophic growth response. For example, activation of ERK or c-Myc is sufficient to induce both pol III transcription and hypertrophic growth in terminally differentiated cardiomyocytes. Moreover, ERK inhibition partially diminishes the activation of both pol III transcription and protein synthesis by hypertrophic stimuli. Evidence for a role of RB in regulating hypertrophy is limited. However, the existing data (WR MacLellan, unpublished) from cardiomyocyte-specific RB-deficient mice suggest that RB attenuates hypertrophic growth. The effects of RB loss on hypertrophy closely parallel the effects on pol III transcription. Therefore, the pol III transcription machinery appears to be a common downstream target of various proteins involved in the regulation of hypertrophy, strongly indicating the functional relevance of pol III transcriptional control during this growth response.

ERK directly phosphorylates TFIIIB in fibroblasts, and thus facilitates the rapid induction of pol III transcription following mitogenic stimulation (Felton-Edkins et al, 2003a). Phosphorylation of TFIIIB by ERK probably also participates in the early activation of pol III transcription in cardiomyocytes. However, further studies are required to confirm this. Other more indirect mechanisms are also likely to contribute to the ERK-mediated induction of class III gene expression,

as ERK has numerous cellular targets that may impinge on the pol III transcription machinery. For example, c-Myc is known to be a downstream transcription factor target of ERK in certain cell types (Cheng et al, 1999; Garrington and Johnson, 1999; Sears et al, 2000). This study provides the first demonstration that c-Myc can be induced by ERK activation in cardiomyocytes (Figure 5.13). Given that c-Myc promotes pol III transcription and hypertrophic growth, this is likely to represent a functionally relevant step in the induction of these processes by ERK. However, ERK inhibition does not prevent c-Myc induction (Figure 5.14), thus questioning the importance of c-Myc activation by ERK. As mentioned in the introduction to this chapter, the regulation of c-Myc expression is highly complex, and not fully understood. The effects of ERK inhibition on c-Myc levels were examined after 16 hours of serum-stimulation. Potentially, c-Myc accumulation is only ERK-dependent at earlier timepoints, and other signalling pathways may determine the level of c-Myc at this later timepoint. A more thorough examination of c-Myc induction by ERK at various times following hypertrophic stimulation would be valuable. Introducing siRNA to c-Myc into cardiomyocytes in the presence of the CAMEK adenovirus may allow the involvement of c-Myc in the induction of pol III transcription by ERK to be tested more directly.

Another downstream target of ERK in cardiomyocytes is mTOR (mammalian target of rapamycin) (Iijima et al, 2002; Proud, 2004; Wang and Proud, 2002b). This protein is involved in controlling the rate of translation in response to nutrients and growth factors in mammalian cells (Huang and Houghton, 2003; Shamji et al, 2003), and is thought to promote cardiomyocyte hypertrophy

(Boluyt et al, 1997; McMullen et al, 2004; Sadoshima and Izumo, 1995; Shioi et al, 2003). The yeast homologue of mTOR (known simply as TOR) activates pol III transcription under favourable growth conditions (Zaragoza et al, 1998). Likewise, there is evidence to suggest that mTOR can promote pol III transcription in proliferating mammalian cells (Graham, White and Scott, unpublished results). Therefore, mTOR-dependent activation of pol III transcription may represent an additional indirect mechanism by which ERK could activate class III gene expression in cardiomyocytes.

The transcription factor c-Myc activates class III gene expression directly in proliferating cells (Gomez-Roman et al, 2003). Further analyses are required to determine whether this is also the case in cardiomyocytes. In an attempt to explore this possibility in the current study, ChIP assays were performed. However, as discussed previously, these assays were unsuccessful, possibly due to inefficient c-Myc immunoprecipitation (as suggested by the failure to detect c-Myc association with the positive control nucleolin promoter). Previous studies have used ChIP assays to demonstrate endogenous c-Myc binding to 5S rRNA and tRNA promoters. However, these analyses were performed using immortalised cell lines (Felton-Edkins et al, 2003b; Gomez-Roman et al, 2003), in which the level of c-Myc is likely to be higher than in the primary, terminally differentiated cells used in this study. Therefore, a more sensitive assay may be required to examine c-Myc promoter occupancy in cardiomyocytes.

Inactivation of RB enhances the hypertrophy-associated production of pol III transcripts in the heart, suggesting that, in addition to its established role as a

negative regulator of class III gene expression in proliferating cells (Scott et al, 2001; Sutcliffe et al, 2000; White et al, 1996), RB is also involved in restraining pol III transcription in terminally differentiated cardiomyocytes. However, unlike the situation in fibroblasts, where RB inactivation increases pol III transcription in both the presence and absence of growth factors (Scott et al, 2001), in cardiomyocytes, the effects of RB loss are only apparent under hypertrophic, not basal, conditions (see Figure 5.10). This discrepancy may be due to the fact that RB inactivation has only been investigated in cultured fibroblasts, whereas the effects of RB loss from cardiomyocytes were studied *in situ*. The more prolonged lack of RB during heart development may invoke mechanisms to compensate for the RB deficiency. It would be interesting to compare acute RB loss in different cell types more directly. This could be achieved by analysing the effects of RB knock down in culture using siRNA. It would also be informative to compare the effects of RB inactivation in a range of different tissue types.

Alternatively, the differential sensitivity of cardiomyocytes and fibroblasts to RB inactivation under basal conditions may reflect cell-type specific differences in the control of pol III transcription by RB. For example, the abundance of TFIIIB, which is the primary target of RB-mediated pol III transcriptional repression (Chu et al, 1997; Larminie et al, 1997), may determine the contribution RB makes to the regulation of pol III transcription. The level of the TFIIIB subunit Brf1 is relatively low in unstimulated cardiomyocytes, compared to fibroblasts, as discussed in Chapter 4. Therefore, whereas sequestration of TFIIIB by RB is necessary for the effective repression of pol III transcription in unstimulated fibroblasts (Scott et al, 2001), insufficient levels of functional TFIIIB may

eliminate the requirement for RB under basal conditions in cardiomyocytes. Consistent with this model, Brf1 levels increase following hypertrophic stimulation (Figures 4.4 and 4.7) and accordingly, RB is required to restrain pol III transcription under such circumstances.

As an alternative approach to investigate the involvement of RB in pol III transcriptional control, the CKI $p16^{INK4a}$ was used to prevent the serum-stimulated inactivation (phosphorylation) of endogenous RB, and thus determine whether active (hypophosphorylated) RB is sufficient to repress pol III transcription in cardiomyocytes. However, $p16^{INK4a}$ expression did not influence the induction of class III gene expression by FCS, thus indicating that active RB is not sufficient to override the effects of positive pol III transcriptional regulators, such as ERK and c-Myc, and repress pol III transcription in these cells (Figure 5.12). However, this conclusion is based on the assumption that $p16^{INK4a}$ does effectively block RB inactivation. As an indicator of RB inactivation, threonine 826 phosphorylation was measured. The phosphorylation of this residue, which is specifically targeted by cyclin D-dependent kinases 4 and 6 (Zarkowska and Mittnacht, 1997), is markedly diminished in the presence of $p16^{INK4a}$ (Figure 5.12). Cyclin D-dependent RB phosphorylation is commonly considered to be a necessary prerequisite for subsequent phosphorylation by cyclin E- and A-dependent kinases (Harbour et al, 1999; Lundberg and Weinberg, 1998). Therefore, it was presumed that $p16^{INK4a}$ would block RB phosphorylation, not only by CDK4/6, but also by the cyclin E/A-dependent kinase CDK2, which is involved in rendering RB inactive with respect to pol III repression (Scott et al, 2001). However, this dogma of sequential RB phosphorylation has recently been

challenged: mouse embryonic fibroblasts derived from knockout mice lacking D-type cyclins, or both CDK4 and 6, still show CDK2-dependent RB phosphorylation, and this appears to be sufficient for RB inactivation and cell cycle progression (Kozar et al, 2004; Malumbres et al, 2004; reviewed by Pagano and Jackson, 2004). Thus, conceivably, even in the presence of $p16^{INK4a}$, RB may still become phosphorylated on several sites following hypertrophic stimulation, leading to its dissociation from TFIIIB, and the resulting activation of pol III transcription. A more extensive comparison of RB phosphorylation in the presence and absence of $p16^{INK4a}$ would allow these issues to be addressed. Co-immunoprecipitation experiments are also required to confirm an interaction between TFIIIB and RB in these cells, and to determine whether this interaction is responsive to different cellular conditions.

Thus, RB appears to repress pol III transcription in cardiomyocytes. However, there may be differences in the control of pol III transcription by RB in cardiomyocytes and undifferentiated fibroblasts, as discussed above. This highlights the importance of characterising cellular control mechanisms in different cell types and *in situ*, rather than assuming that mechanisms operating in cultured fibroblasts will be generally applicable. Further investigation is required to fully elucidate the role of RB in regulating class III gene expression in terminally differentiated cardiomyocytes. Such studies will likely make a valuable contribution to the limited understanding of RB targets in different cell types, and the involvement of this protein in cell growth control.

In summary, the results presented in this chapter provide a mechanistic link between the activation of positive regulators of hypertrophy (ERK and c-Myc), and enhanced translational capacity, through increased pol III transcription. Additionally, recent findings implicating RB as a negative regulator of hypertrophy (MacLellan et al, unpublished results) can be at least partly explained by its ability to restrict class III gene expression in the heart. Not only do these findings contribute to the current understanding of processes governing hypertrophy, but also indicate that proliferating and terminally differentiated cells utilise common mechanisms to regulate pol III transcription and cell growth. However, there appear to be some cell-type specific differences in these shared control mechanisms, which warrant further investigation. The following chapter explores the regulation of Brf1 expression in cardiomyocytes, and considers the possibility that the induction of this essential TFIIIB component is involved in activating pol III transcription during the hypertrophic growth of cardiomyocytes.

CHAPTER 6
Regulation of pol III transcription by changes in Brf1 expression in cardiomyocytes

6.1 Introduction

Brf1 along with TBP and Bdp1 comprise the pol III-specific transcription factor TFIIIB. Brf1 is a critical determinant of the integrity of TFIIIB: Brf1 and TBP interact directly to form a stable complex, with which Bdp1 is weakly associated in a Brf1-dependent manner (Geiduschek and Kassavetis, 2001). As discussed in Chapter 1, the primary purposes of TFIIIB are to recruit pol III to the appropriate promoters, as determined by TFIIIC, and to facilitate promoter opening. Brf1 is essential for both of these TFIIIB functions. For example, Brf1 mediates the initial interaction between TFIIIB and promoter-bound TFIIIC, which, at least in yeast, is the rate-limiting step for pol III transcription (Geiduschek and Kassavetis, 2001; Moir et al, 1997; Rameau et al, 1994; Schramm and Hernandez, 2002). In addition, Brf1 makes direct contacts with pol III itself, thus participating in polymerase recruitment, and is necessary for the subsequent formation of an open promoter structure, competent for transcription initiation (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). Given these central roles of Brf1 in TFIIIB function, it is perhaps not surprising that changes in class III gene expression are often achieved through the regulation of this subunit, as discussed below. In fact, Brf1 is the limiting factor for pol III transcription under various circumstances in yeast and mammalian cells (Fairley et al, 2003; Felton-Edkins et al, 2003a; López-de-León et al, 1992; Sethy et al, 1995).

In *S. cerevisiae*, pol III transcription declines during the transition from logarithmic to stationary phase growth. This results from a specific reduction in the level, and possibly the activity, of Brf1 (Sethy et al, 1995). Similarly, in mammalian cells, decreases in Brf1 abundance contribute to the down-regulation of pol III transcription that occurs as F9 cells differentiate (Alzuherri and White, 1998) and during p53-induced sustained cell cycle arrest (Eichhorn and Jackson, 2001).

The activity of mammalian Brf1 is also regulated by phosphorylation (Fairley et al, 2003; Felton-Edkins et al, 2003a; Johnston et al, 2002). In cycling cells, pol III transcription increases during late G1 and declines during mitosis (White et al, 1995b). Although the level of Brf1 remains constant throughout the cell cycle (Scott et al, 2001; Fairley et al, 2003), phosphorylation of this factor plays a key role in mediating these fluctuations in class III gene expression. For example, Brf1 becomes phosphorylated when quiescent fibroblasts are induced to enter the cell cycle by serum-stimulation (Johnston et al, 2002). This is at least partly mediated by ERK and CK2 (Felton-Edkins et al, 2003a; Johnston et al, 2002), kinases that promote cell growth and division. Phosphorylation of Brf1 by either of these kinases increases the association of TFIIIB with TFIIIC and, in the case of ERK-mediated phosphorylation, TFIIIB with pol III, thus promoting transcription initiation (Felton-Edkins et al, 2003a; Johnston et al, 2002). Brf1 is also phosphorylated during mitosis (Fairley et al, 2003). However, this modification is thought to disrupt the TFIIIB complex and thus repress, rather than activate, pol III transcription (Fairley et al, 2003). The kinase(s) responsible for this mitotic Brf1 phosphorylation have yet to be identified.

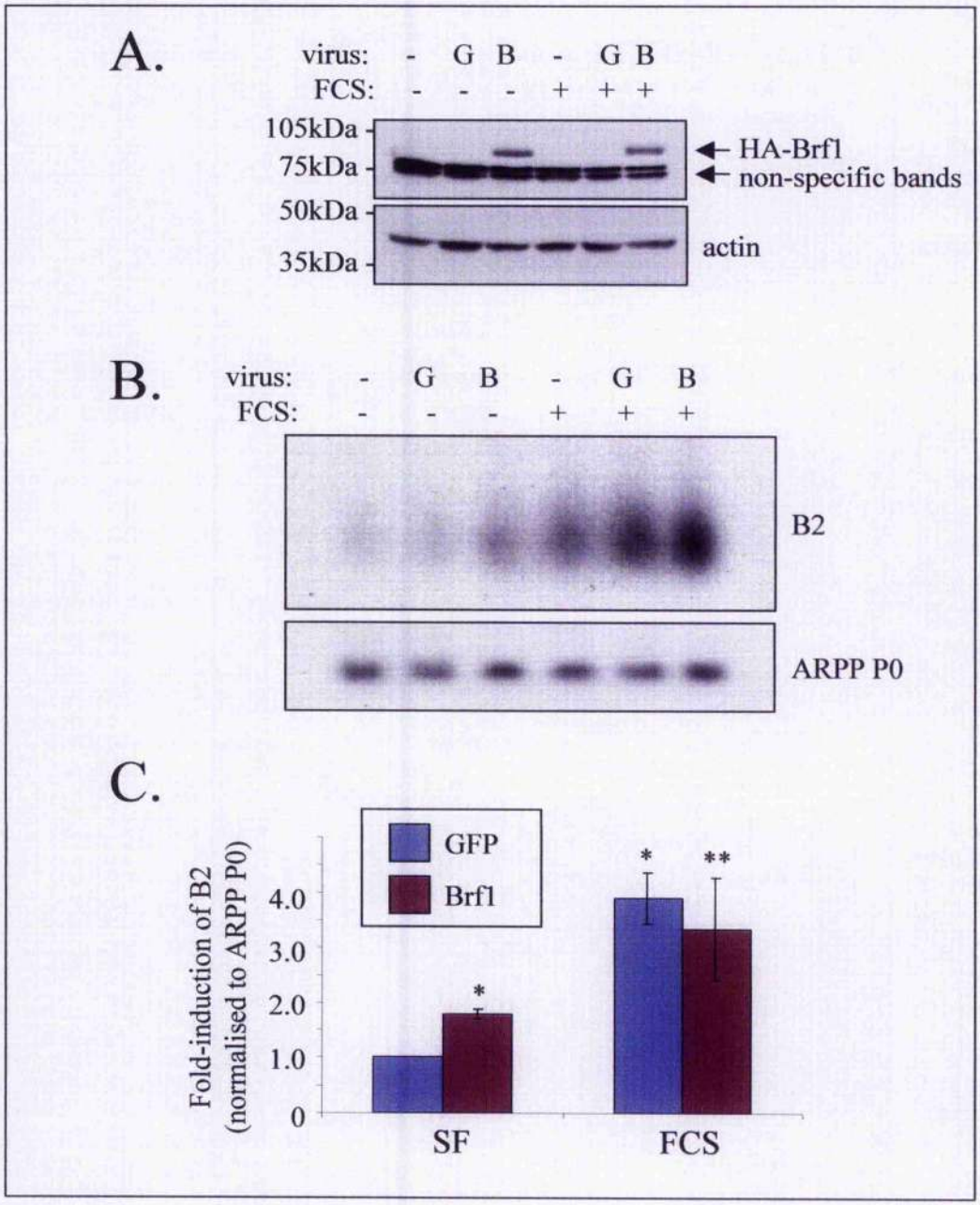
Therefore, regulation of the abundance and/or activity of this pivotal TFIIIB subunit can promote or disrupt critical interactions between components of the pol III transcription machinery and thereby activate or repress pol III transcription according to cellular requirements. The level of Brf1 increases in response to the hypertrophic stimulation of cardiomyocytes, as shown in Chapter 4. Given the critical role that Brf1 plays in determining the rate of pol III transcription in other eukaryotic systems, it is likely that this elevated Brf1 abundance will contribute to the enhanced class III gene expression that accompanies hypertrophic growth. This possibility is addressed in the current chapter, along with the mechanisms regulating the expression of this transcription factor.

6.2 Results

6.2.1 Brf1 is limiting for pol III transcription in unstimulated cardiomyocytes

To determine whether the increase in Brf1 levels following hypertrophic stimulation of cardiomyocytes contributes to the activation of pol III transcription, the effects of raising the concentration of this TFIIIB subunit were assessed. Cultured cardiomyocytes were infected with adenoviruses expressing GFP alone, as a negative control, or HA-Brf1. Western analysis of protein lysates derived from these cells confirmed HA-Brf1 overexpression where appropriate (Figure 6.1A). To analyse the effects of increasing the level of Brf1 on pol III transcription, Northern blotting was performed. As shown in Figures 6.1B and C, enhanced Brf1 expression causes a significant induction of B2 transcripts (1.8-fold), compared to control infected cells, in the absence of hypertrophic stimuli. This indicates that Brf1 is limiting for pol III transcription under basal conditions in terminally differentiated cardiomyocytes. Although in Figure 6.1B, B2 also seems to be elevated in serum-stimulated cells expressing HA-Brf1, compared to those expressing GFP alone, when normalised to ARPP P0 in three separate experiments, it was apparent that B2 abundance does not increase significantly under these conditions (as displayed in Figure 6.1C). Therefore, in contrast to the situation in unstimulated cardiomyocytes, it was concluded that following hypertrophic stimulation, Brf1 overexpression does not cause any further enhancement of pol III transcription (Figure 6.1C). This suggests that the level of Brf1 no longer restricts class III gene expression, presumably due to the hypertrophy-associated increase in the abundance of this protein. These data are consistent with a model whereby enhanced Brf1 expression promotes the

Figure 6.1: Brf1 induces pol III transcription in unstimulated cardiomyocytes. Cultured cardiomyocytes were infected with adenoviruses expressing HA-Brf1 (B) or GFP (G) alone. Cells were serum-starved or stimulated with 10% FCS for 16 hours prior to extracting whole cell protein or RNA. A. Western blotting was performed using anti-HA and anti-actin antibodies. B. RNA was analysed by Northern blotting. The upper panel shows the blot probed for B2, and the lower panel shows the same blot stripped and re-probed for ARPP P0. C. Northern blots were quantified by densitometry, and B2 levels normalised to ARPP P0. This was done for 3 separate experiments. The average fold increases obtained, along with standard deviations from the means, are represented in the graph (*significantly different from SF GFP, $p<0.05$; **not significantly different from FCS GFP). The viruses used for these analyses were prepared by Dr F Cairns, University of Glasgow, UK.


A.
virus: - G B - G B
FCS: - - - + + +
105kDa
75kDa
50kDa
35kDa
HA-Brf1
non-specific bands
actin
B.
virus: - G B - G B
FCS: - - - + + +
B2
ARPP P0
C.
Fold-induction of B2
(normalised to ARPP P0)
GFP
Brf1
4.0
3.0
2.0
1.0
0
*
*
**
SF
FCS

formation of functional TFIIIB complexes, which can then participate in the activation of pol III transcription during hypertrophic growth (Figure 6.2). However, overexpressing Brf1 in unstimulated cardiomyocytes is not sufficient to induce pol III transcription to the same extent as FCS (Figure 6.1C). This suggests that other proteins, such as ERK and c-Myc, also play critical roles in activating the pol III transcription machinery in response to hypertrophic stimulation, consistent with the data presented in Chapter 5.

6.2.2 The regulation of Brf1 expression in cardiomyocytes

Having determined that an increase in Brf1 abundance is likely to be a functionally relevant step contributing to the activation of pol III transcription during hypertrophic growth, the mechanisms controlling this enhanced expression were investigated. Potentially, Brf1 levels could be regulated at the level of transcription, mRNA processing, translation, or post-translationally through the control of protein stability. Only two previous studies have reported changes in Brf1 abundance as a means for regulating pol III transcription in mammalian cells (Alzuherri and White, 1998; Eichhorn and Jackson, 2001). In the latter of these studies, Brf1 levels were found to be controlled by ubiquitin-mediated proteolysis. The ubiquitin-proteasome pathway is a common means of regulating the function of numerous proteins involved in fundamental cellular processes including the cell cycle, differentiation and development, stress responses, DNA repair and apoptosis (Ciechanover, 1998; Jentsch and Schlenker, 1995; Weissman, 2001). Eichhorn and Jackson (2001) demonstrated that Brf1 destabilisation contributes to the decline in pol III transcription associated with a

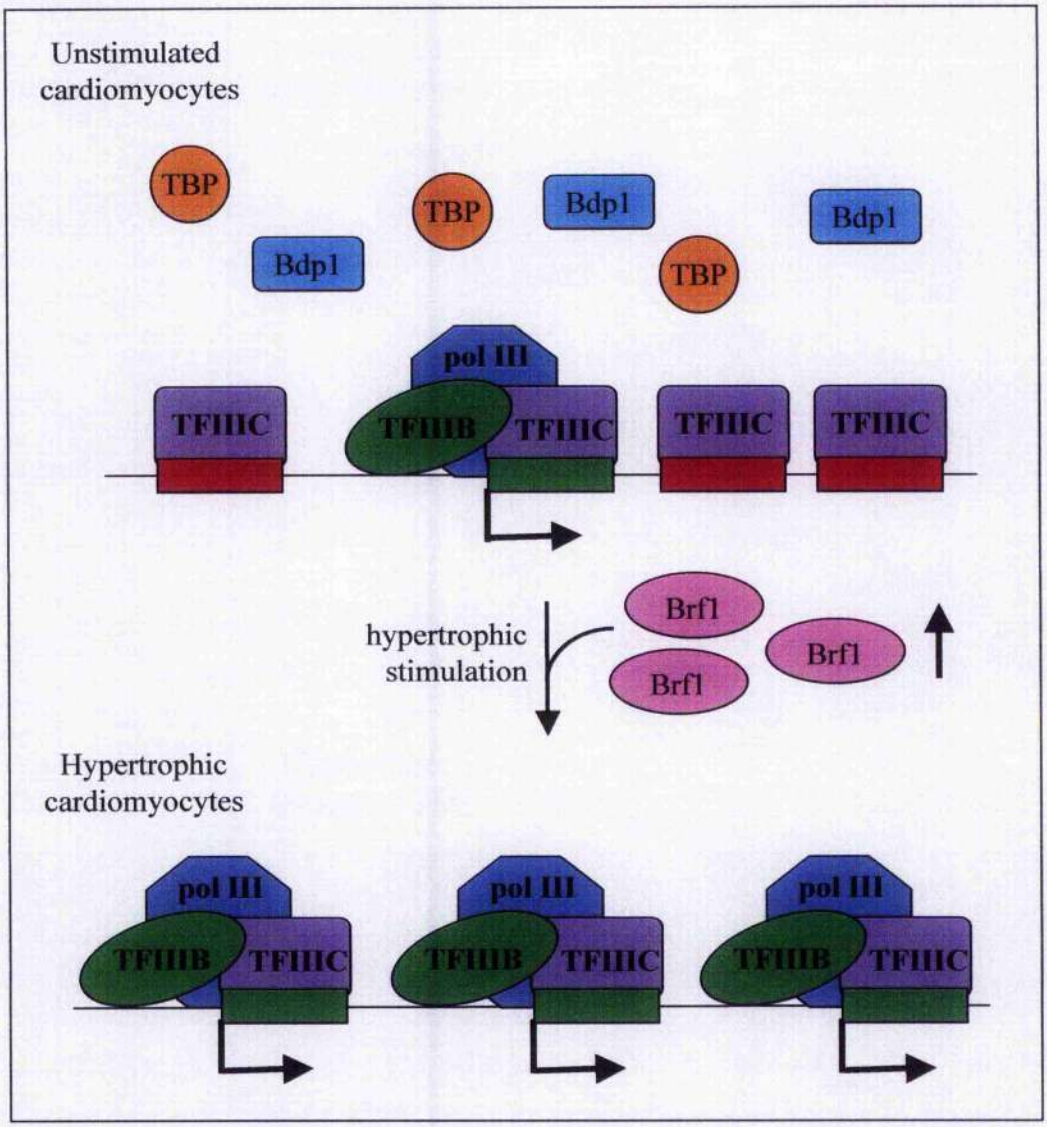


Figure 6.2: Model depicting Brf1-mediated activation of class III gene expression during cardiomyocyte hypertrophy. Low levels of Brf1 in unstimulated cardiomyocytes are likely to restrict the formation of TFIIIB complexes, and thereby limit the rate of pol III transcription. Accordingly, increasing the level of Brf1 in these cells promotes pol III transcription. Thus, the hypertrophic stimulation of cardiomyocytes is likely to activate class III gene expression by enhancing the availability of this limiting factor. Inactive class III genes are represented by red boxes, whereas green boxes indicate active genes.

sustained, p53-induced, cell cycle arrest. Brf1 levels and pol III transcription could be restored in these arrested cells by inhibition of the proteasome. To test whether rapid protein turnover accounts for the low level of Brf1 in unstimulated cardiomyocytes, the proteasome inhibitor MG132 was employed. Cardiomyocytes were cultured in the absence of hypertrophic stimuli and, 4 hours before harvesting, MG132 or vehicle (0.1% DMSO) were added to the cells. Protein lysates were analysed by Western blotting. To ensure MG132 efficacy, the effect of this proteasome inhibitor on the level of cyclin D1, which is known to have a short half-life due to rapid proteasome-mediated turnover (Diehl et al, 1997), was assessed. As shown in Figure 6.3 (middle panel), MG132 caused a substantial accumulation of cyclin D1, thus confirming proteasome inhibition. However, Brf1 levels were unaffected, suggesting that this protein is relatively stable in terminally differentiated cardiomyocytes (Figure 6.3, top panel). Therefore, ubiquitin-mediated proteolysis does not appear to regulate Brf1 in these cells.

The most common means for controlling gene expression is through the regulation of transcription (White, 2001). To investigate whether hypertrophic stimuli increase the production of transcripts encoding Brf1, the level of Brf1 mRNA was analysed by RT-PCR. Figure 6.4 shows that, along with the previously described increase in ANF and tRNA transcripts (Figures 3.4 and 3.7, respectively), hypertrophic stimulation also increases the level of Brf1 mRNA. ARPP P0 levels were unaffected, demonstrating the specificity of these effects. Thus, although the elevation in Brf1 transcripts may be due to an increase in

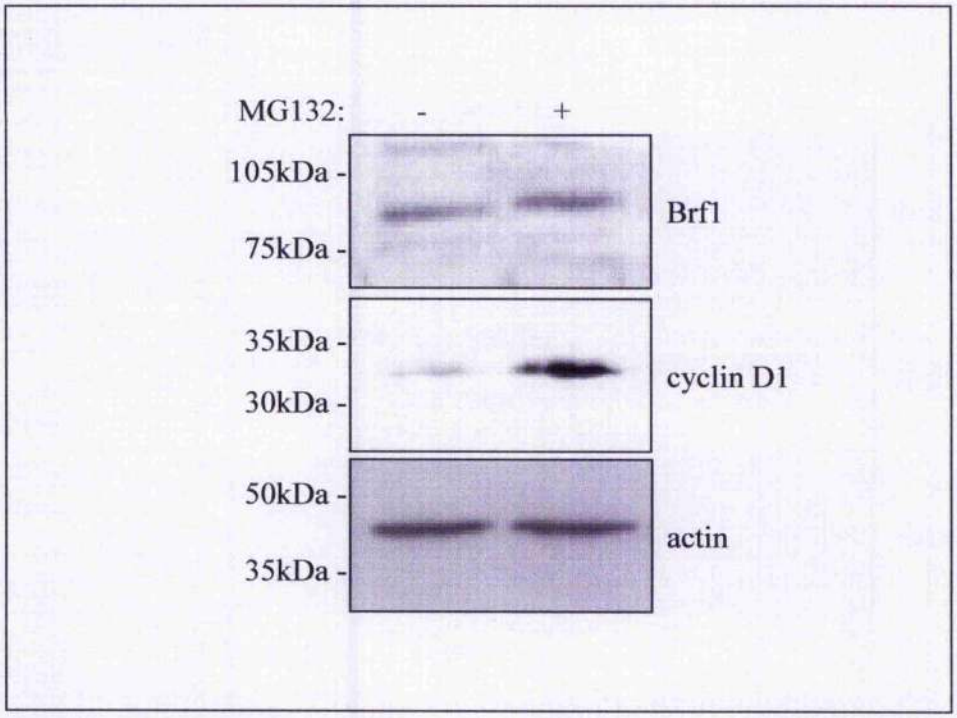


Figure 6.3: Brf1 levels are not regulated by proteasome-mediated turnover in cardiomyocytes. Cardiomyocytes were cultured in serum-free medium. 4 hours before harvesting, 10 μM MG132 or vehicle (0.1% DMSO) were added to the cells. 75 μg of protein lysates were then resolved by SDS-PAGE, and Western blotting was performed with antibodies against the proteins indicated.

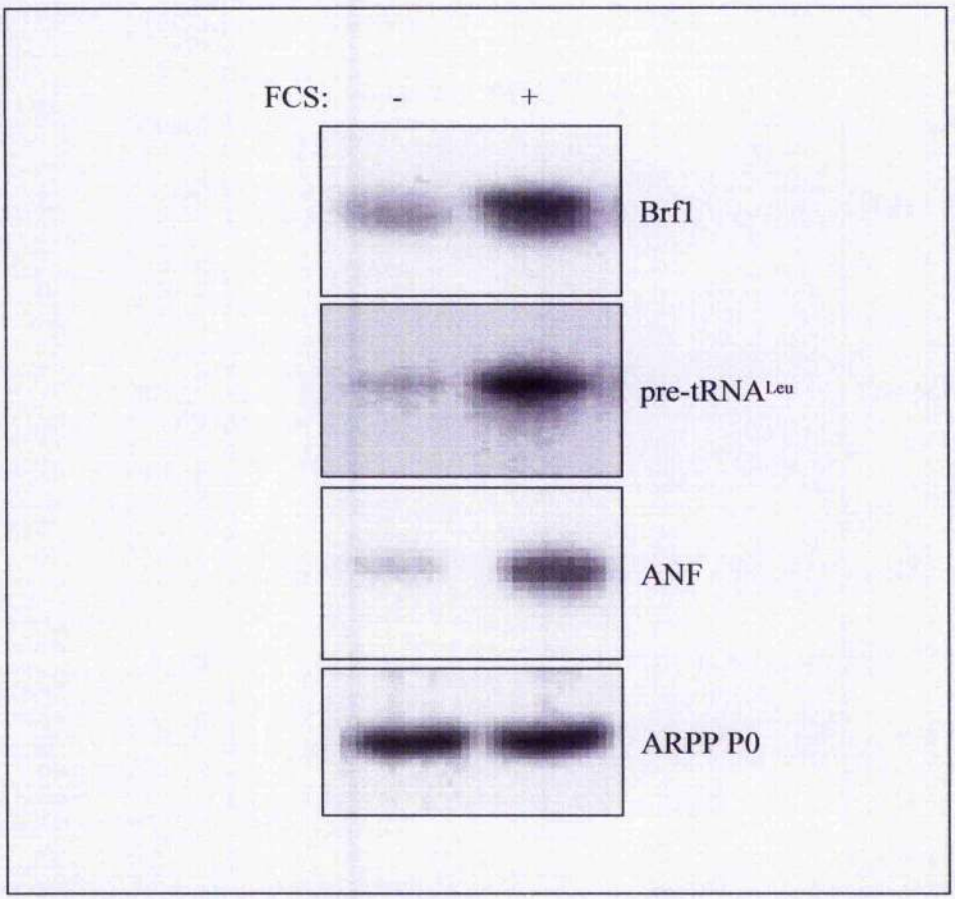


Figure 6.4: Hypertrophic stimulation of cardiomyocytes increases Brf1 at the mRNA level. Cultured cardiomyocytes were serum-starved for 24 hours, then either maintained in serum-free media for a further 16 hours, or exposed to 10% FCS. Total RNA was extracted from these cells and 3 μg used to generate cDNAs by reverse transcription. cDNAs were then amplified by PCR using primers specific for Brf1, pre-tRNA^{Leu}, ANF or ARPP P0, as indicated.

mRNA stability, it most likely indicates that hypertrophic stimuli enhance Brf1 expression by activating Brf1 gene transcription.

As discussed in Chapter 3, hypertrophic growth is accompanied by characteristic changes in the transcription of several class II genes, including the induction of immediate early genes, the re-expression of certain foetal genes, and the upregulation of constitutively expressed contractile proteins (Figure 3.1). Many of these alterations in gene expression are mediated by ERK (Bueno et al, 2000; Gillespie-Brown et al, 1995; Glennon, et al, 1996; Harris et al, 2004; Ueyama et al, 2000; Yue et al, 2000), which phosphorylates a range of nuclear transcription factors (Babu et al, 2000; Bueno and Molkentin, 2002; Liang et al, 2001; Morimoto et al, 2000). Given that ERK can also activate class III gene expression (as demonstrated in Chapter 5), for which Brf1 is essential, the involvement of this kinase in the induction of Brf1 was investigated.

To explore the involvement of ERK in the induction of Brf1 gene transcription, the effects of ERK activation on the level of Brf1 transcripts were examined. Cultured cardiomyocytes were infected with control or CAMEK-expressing adenoviruses in the absence of hypertrophic stimuli. RT-PCR analysis, using RNA extracted from these cells, demonstrated that ERK activation does indeed induce Brf1 at the mRNA level (Figure 6.5). ERK activation was also accompanied by an increase in tRNA transcripts, as found previously (Figure 5.6). The level of the negative control mRNA (ARPP P0) was unchanged, confirming that these effects are specific. Thus, the Brf1 gene is likely to

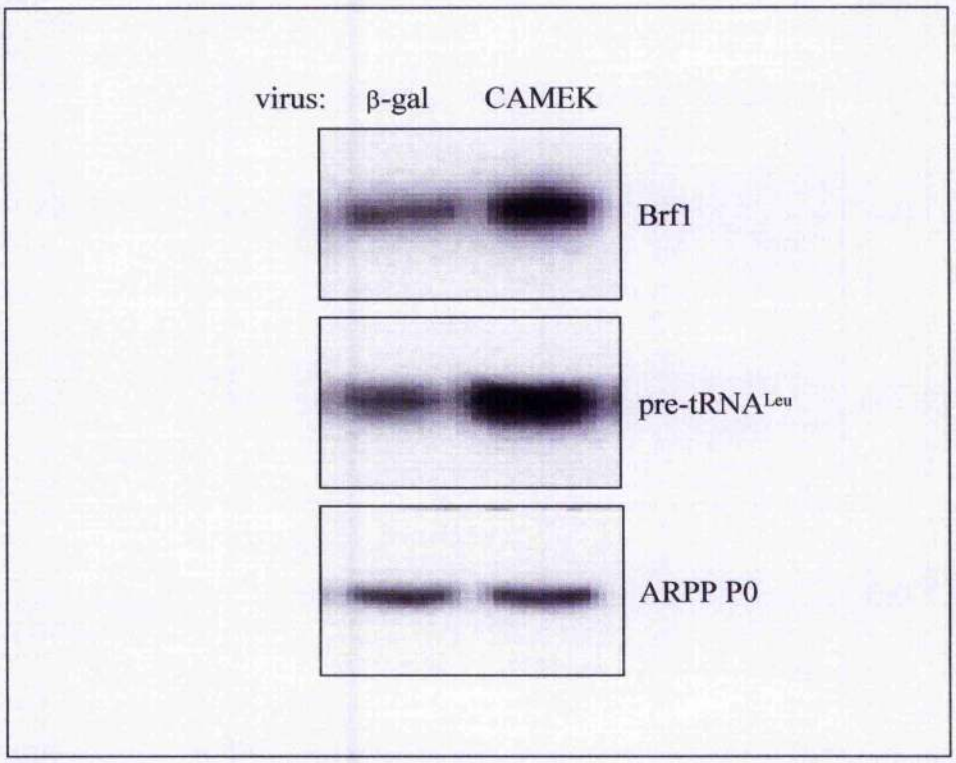


Figure 6.5: ERK induces Brf1 at the mRNA level in cardiomyocytes. Cultured cardiomyocytes were infected with equivalent amounts of adenovirus expressing CAMEK or β–gal (negative control), as indicated. Cells were serum starved for 48 hours prior to extracting whole cell RNA. 3 μg of this RNA were used to generate cDNAs by reverse transcription. cDNAs were then amplified by PCR using Brf1-, pre-tRNA^{Leu}-, or ARPP P0-specific primers, as specified. The viruses used for these analyses were a kind gift from Dr JD Molkentin, University of Cincinnati, USA.

represent an additional transcriptional target of the ERK pathway in cardiomyocytes.

For this induction of Brf1 mRNA by ERK to be functionally relevant, an increase in the abundance of the Brf1 protein must also occur. Western analysis confirmed that ERK activation does induce Brf1 at the protein level (Figure 6.6), and in addition, this effect is clearly specific to the Brf1 subunit of TFIIIB, as TBP and Bdp1 abundance do not respond to ERK activation. Therefore, given that Brf1 is limiting for pol III transcription in the absence of hypertrophic stimuli, this induction of Brf1 expression is likely to play a critical role in the activation of pol III transcription, and perhaps cell growth, by ERK in cardiomyocytes.

To determine whether ERK activation is necessary for the induction of Brf1 by hypertrophic stimuli, the MEK inhibitor PD98059 was used. This inhibitor attenuates the induction of both pol III transcription and protein synthesis by FCS in cardiomyocytes (Figures 5.4 and 5.5). As shown in Figure 6.7, blocking ERK activation also reduces the FCS-induced increase in Brf1 expression. This provides further evidence to support the proposed involvement of ERK in the induction of Brf1 during hypertrophic growth. However, ERK inhibition does not completely abrogate Brf1 induction, suggesting that other ERK-independent pathways also ensure that Brf1 expression is increased during cardiomyocyte hypertrophy.

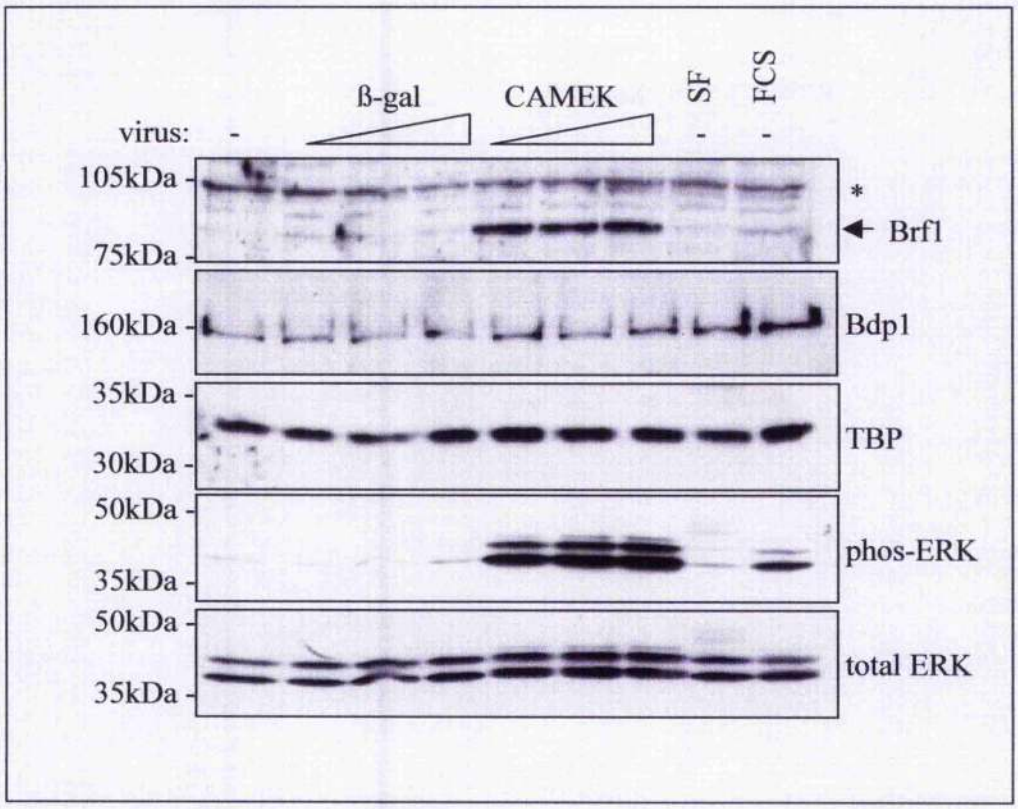


Figure 6.6: ERK induces Brf1 at the protein level in cardiomyocytes. Cultured cardiomyocytes were infected with increasing amounts of adenovirus expressing CAMEK or β–gal (negative control), as indicated. Cells were serum starved for 48 hours before extracting whole cell protein. For comparison, uninfected cells were incubated without (SF) or with 10% FCS for 16 hours. 75 μg of protein were resolved by SDS-PAGE and Western blotting was performed using the antibodies specified. The viruses used for these analyses were a kind gift from Dr JD Molkentin, University of Cincinnati, USA.

*indicates a non-specific band.

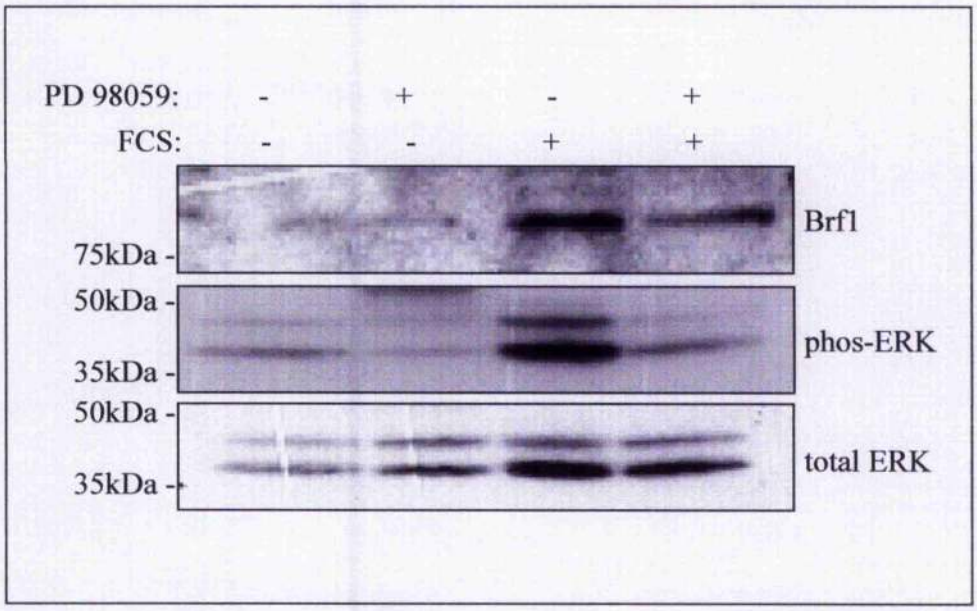


Figure 6.7: ERK inhibition attenuates the hypertrophy-associated increase in Brf1. Cultured cardiomyocytes were serum-starved for 24 hours, then either maintained in serum-free medium, or exposed to 10% FCS, for 16 hours in the presence of 50 μM PD98059 or vehicle (0.1% DMSO), as indicated. 75 μg of protein were resolved by SDS-PAGE and Western blotting was performed using the antibodies specified.

6.3 Discussion

The induction of hypertrophic growth is accompanied by enhanced Brf1 expression. The data presented in this chapter imply that this specific increase in Brf1 abundance contributes to the activation of pol III transcription during hypertrophy: in the absence of hypertrophic stimuli, pol III transcription can be induced simply by raising the level of Brf1, suggesting that this TFIIIB subunit is rate-limiting for pol III transcription. Furthermore, the fact that hypertrophic cardiomyocytes do not respond to exogenous Brf1 suggests that the hypertrophy-associated increase in the expression of this factor is sufficient to permit maximum levels of class III gene expression. This is the first indication that pol III transcription can be activated by a growth factor-dependent increase in Brf1 levels in mammalian cells. Studies aimed at determining the mechanisms regulating pol III transcription in other differentiated cell types may reveal that controlling Brf1 abundance is a common means of determining pol III transcriptional output following terminal differentiation.

Data presented in Chapter 5 demonstrate that ERK induces class III gene expression in cardiomyocytes. However, the mechanisms contributing to this induction were not determined. The results described in this chapter provide a mechanistic link between ERK activation and enhanced pol III transcription, through the induction of Brf1: active ERK can increase Brf1 at the mRNA and protein levels in the absence of hypertrophic stimuli; in addition, the full induction of Brf1 by FCS is dependent on ERK activation. ERK-regulated Brf1 expression has not previously been reported, but is likely to make an important functional contribution to the induction of class III gene transcription, and hence

hypertrophic growth, in cardiomyocytes. Again, examination of a variety of cell types is required to determine whether this ERK-mediated induction of Brf1 expression is cardiomyocyte-specific, or a more generally employed mechanism for controlling pol III transcription. Another instance where ERK could potentially be involved in the induction of Brf1 is discussed below.

HPV16 is a highly oncogenic strain of the human papillomavirus (HPV), and is a common cause of cervical cancer (zur Hausen, 2000). Although Brf1 induction has not previously been shown to accompany normal cell growth or division, Brf1 levels were found to be specifically elevated in HPV16-transformed cervical epithelium, and this is thought to contribute to the abnormally high level of pol III transcripts found in tumours of the cervix (Daly et al, 2004, *In press*). HPV16 oncoproteins have also been associated with aberrant activation of the ERK signalling pathway (Chakrabarti et al, 2004; Gu and Matlashewski, 1995). Thus, the finding that ERK can enhance Brf1 expression provides a possible explanation for this HPV-induced increase in Brf1 abundance, and therefore may be of relevance, not only to the hypertrophic growth of cardiomyocytes, but also to the development of this prevalent human cancer.

Although Brf1 induction is likely to facilitate the activation of pol III transcription during hypertrophic growth, increasing Brf1 alone is insufficient to attain levels of pol III transcription comparable to those induced by FCS (Figure 6.1C). Furthermore, Brf1 protein levels do not significantly increase until 3 to 6 hours after hypertrophic stimulation, whereas pol III transcription is activated after only 1 to 2 hours (Figures 4.7 and 4.5, respectively). Therefore, additional

mechanisms must also contribute to pol III transcriptional regulation in cardiomyocytes. This is consistent with the data presented in Chapter 5, which suggest that multiple pathways are likely to converge on the pol III transcription machinery. For instance, c-Myc can positively regulate pol III transcription in cardiomyocytes, and this is likely to participate in the initial activation of class III gene expression. In addition, although the induction of Brf1 by ERK is likely to partly explain the ability of this kinase to promote pol III transcription, it is probable that direct, ERK-mediated phosphorylation of Brf1, as occurs in fibroblasts (Felton-Edkins et al, 2003a), will also be involved, thus allowing a rapid response. Further investigations are required to confirm these possibilities. ERK is thought to phosphorylate Brf1 on threonine 145 (Felton-Edkins et al, 2003a). Mutating this residue to an aspartate would mimic such phosphorylation. If ERK-directed Brf1 phosphorylation does contribute to pol III transcriptional induction in cardiomyocytes, adenoviral-mediated expression of this mutant would be expected to cause a more robust activation of pol III transcription in unstimulated cells than wild-type Brf1.

The increase in Brf1 mRNA, in response to hypertrophic stimulation, implies an increase in Brf1 gene transcription, although post-transcriptional control is also possible. To gain some insight into the transcription factors potentially involved in this regulation, the upstream region of the Brf1 gene was analysed using the consensus binding site prediction programme *CONSITE* (available at www.phylofoot.org). This identified putative binding sites for various transcription factors known to be involved in determining the hypertrophic phenotype. For example, both the rat and human Brf1 genes contain several

potential c-Myc (E-boxes) and AP-1 binding sites, as shown in Figure 6.8. As discussed previously, c-Myc induces pol III transcription and hypertrophic growth in cardiomyocytes (see Chapter 5). Although c-Myc is likely to directly influence the pol III transcription machinery, as in other cell types (Gomez-Roman et al, 2003), it may also promote pol III transcription more indirectly by increasing the availability of Brf1. AP-1 transcriptional activators are dimers containing members of the Fos and Jun families (White, 2001). c-fos and c-jun are induced by hypertrophic stimuli (Iwaki et al, 1990; Izumo et al, 1988; Komuro et al, 1988; Komuro et al, 1990; Komuro et al, 1991; Sadoshima et al, 1992; Sadoshima and Izumo, 1993a), leading to the transcriptional activation of genes characteristically upregulated during hypertrophy (Bishopric et al, 1992; Herzig et al, 1997; Omura et al, 2002; Paradis et al, 1996). Furthermore, both c-Myc and c-fos induction are regulated by the ERK pathway in cardiomyocytes (Chapter 5, Figure 5.13; Babu et al, 2000; Gillespie-Brown et al, 1995), hence providing a potential connection between ERK activation and Brf1 accumulation. However, c-Myc can be induced independently of ERK activation (Figure 5.14), which may explain the partial FCS-stimulated increase in Brf1 abundance in the presence of the MEK inhibitor PD98059 (Figure 6.7). Thus, it would be interesting to examine the binding of these transcription factors to the Brf1 promoter, and directly test their effects on Brf1 expression.

The abundance of Brf1 is not altered in response to growth stimuli in proliferating cells. This suggests that Brf1 expression is regulated in a cell-type specific manner. MEF2 is a transcription factor whose function is restricted to a few tissue types, including cardiac muscle, and is known to be a crucial regulator

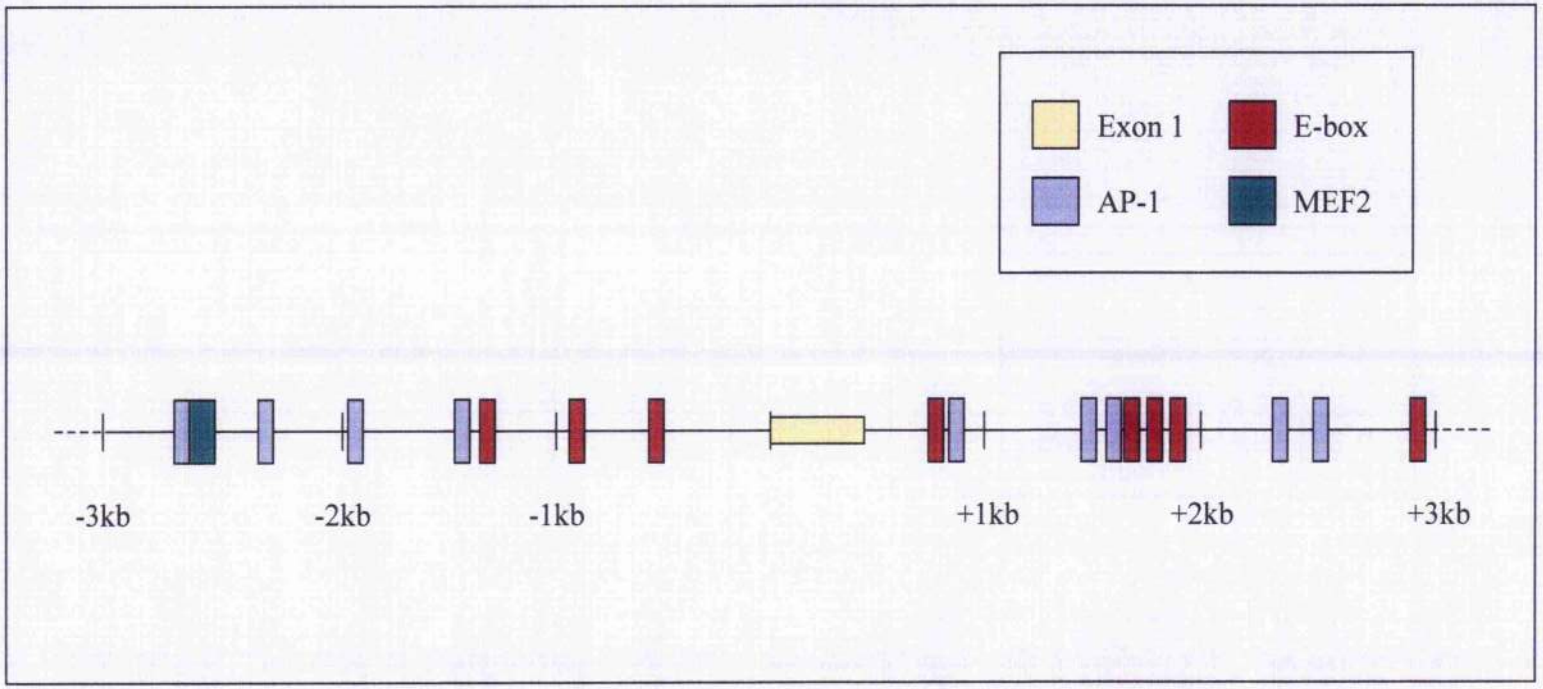


Figure 6.8: Consensus binding sites for transcription factors potentially involved in regulating Brf1 expression. 6kb of human and rat genomic DNA, spanning the upstream end of the Brf1 gene, was analysed using the transcription factor binding site prediction programme *CONSITE* (available at www.phylofoot.org). Potential transcription factor binding sites identified in both species, and their approximate locations, are represented in the diagram, as specified. Only sites for transcription factors with established roles in regulating cardiomyocyte growth are shown.

of cardiomyocyte growth and gene expression (Akazawa and Komuro, 2003; Kolodziejczyk et al, 1999; Molkentin and Markham, 1993; Nadruz et al, 2003). A putative MEF2 binding site was identified upstream of the rat and human Brf1 genes (Figure 6.8), making this factor a candidate for such cell-type specific regulation. In addition, intron 2 of the Brf1 gene contains a consensus binding site for the transcriptional repressor REST (I Wood, personal communication; http://www.bioinformatics.leeds.ac.uk/group/online/RE1db/re1db_home.htm), which is a negative regulator of cardiomyocyte hypertrophy (Kuwahara et al, 2003). Potentially, this transcription factor may be involved in restraining Brf1 expression under basal conditions in terminally differentiated cardiomyocytes. Further analyses of the mechanisms controlling Brf1 expression are likely to uncover links between transcriptional regulators of hypertrophy and changes in protein synthesis.

In summary, the experiments described in this chapter have identified a novel mode for regulating pol III transcription during cell growth. Furthermore, this is the first study to examine the mechanisms regulating the expression of the central TFIIIB subunit Brf1, revealing an important role for the ERK signalling pathway. Further investigations are required to establish whether these mechanisms are a widespread means of controlling pol III transcription, for example during the growth of other differentiated cells or the abnormal proliferation of tumour cells. Future work aims to analyse the effects of blocking the hypertrophy-associated increase in Brf1, using antisense or siRNA technologies, to firmly establish the importance of this enhanced expression in the activation of class III gene expression and hypertrophic cell growth.

CHAPTER 7
Final Discussion

Cardiovascular disease is one of the leading causes of death in industrialised countries, with associated health care costs estimated to reach over £7 billion each year in the UK alone (statistics obtained from the British Heart Foundation: http://www.bhf.org.uk/news/index.asp?secondlevel=241&thirdlevel=751&artID =3362). Therefore, research into the mechanisms regulating heart function is of vital importance in order to improve our understanding of the causes of heart failure. Cardiomyocyte hypertrophy is associated with many cardiovascular disorders (Harjai et al, 1999; Kannel, 1990; Levy et al, 1990), and thus the molecular basis of this process has been the subject of intensive investigation. One of the cardinal features of this hypertrophy is an increase in cell size, without proliferation. However, the mechanisms responsible for this cell growth response have yet to be fully elucidated. In fact, although the mechanisms regulating cell division have attracted a considerable amount of interest over the years, comparatively little is known about the control of cell growth in general. Therefore, cardiomyocyte hypertrophy is not only worthy of study because of its relevance to heart disease, but also because it sheds light on the poorly understood mechanisms underlying cell growth.

Cell growth requires an accumulation of protein. In the case of cardiomyocyte hypertrophy, this is dependent on an increase in translational capacity (Brandenburger et al, 2001). Translational capacity is determined by the availability of numerous molecules involved in biosynthesis, including ribosomal

components, tRNAs and translation factors. Pol III-transcribed genes encode a variety of small, untranslated RNAs with essential roles in cellular biosynthesis (see Chapter 1). Therefore, it was hypothesised that an increase in class III gene expression would accompany cardiomyocyte hypertrophy. This was confirmed by the data presented in Chapter 3, which demonstrate that various hypertrophic stimuli enhance pol III transcription, both in culture and in the heart, leading to an increase in all the class III gene products tested, including 5S rRNA, tRNAs and U6 snRNA. This general increase in class III gene expression represents a previously unreported route to increase protein synthetic capacity, and hence hypertrophic growth, in cardiomyocytes. In addition, this is the first demonstration that pol III transcriptional activation accompanies cell growth independently of cell division. The subsequent chapters of this thesis aimed to elucidate the mechanisms responsible for this regulation. As discussed below, this revealed that several mechanisms contribute to the control of pol III transcription in terminally differentiated cardiomyocytes, suggesting that such regulation is of fundamental importance.

7.1 Mechanisms contributing to the regulation of pol III transcription in cardiomyocytes

The transcription of a class III gene is dependent on pol III recruitment to the appropriate promoter. For the majority of genes transcribed by pol III, this is determined by the transcription factors TFIIIC and TFIIIB, which bind specific promoter sequences and directly recruit the polymerase, respectively (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). ChIP assays

were used to monitor pre-initiation complex assembly on class III genes in response to hypertrophic stimulation. This revealed a specific increase in TFIIIB promoter occupancy on tRNA and 5S rRNA genes, while the association of TFIIIC was unaffected (as illustrated in Figures 4.2 and 7.2). Therefore, it was concluded that polymerase binding, and hence transcription initiation, are regulated at the level of TFIIIB recruitment. This is consistent with the well-recognised role of TFIIIB as a central regulator of pol III transcription in other eukaryotic cell types (Gottesfeld et al, 1994; Sethy et al, 1995; Tower and Sollner-Webb, 1988; White et al, 1989; White et al, 1995a, b; White, 1998). The frequent targeting of TFIIIB by numerous control strategies likely reflects its essential role in various aspects of pol III transcription initiation.

7.1.1 Pol III transcription in cardiomyocytes is positively regulated by c-Myc, ERK and Brf1

TFIIIB function in dividing cells can be regulated through its interaction with various proteins. Some of these proteins have been implicated in controlling hypertrophy; therefore, their involvement in pol III transcriptional regulation was studied in cardiomyocytes. For instance, both ERK and c-Myc are involved in the control of pol III transcription during cellular proliferation (Felton-Edkins et al, 2003a, b; Gomez-Roman et al, 2003), and have previously been shown to promote the hypertrophic growth of heart muscle cells (Bueno et al, 2000; Glennon et al, 1996; Ueyama et al, 2000; Wang and Proud, 2002a; Xiao et al, 2001; Yue et al, 2000). Following hypertrophic stimulation, c-Myc induction and ERK activation precede the initial increase in pol III transcription, as shown in Chapter 4. In addition, data presented in Chapter 5 reveal that c-Myc induction or

ERK activation is sufficient to induce pol III transcription in the absence of hypertrophic stimuli. In the case of c-Myc, this was demonstrated both in culture and in the heart *in situ*. Furthermore, ERK inhibition attenuates the hypertrophy-associated induction of class III gene expression. Thus, this study has uncovered a connection between these molecules and their ability to enhance cardiomyocyte biosynthesis, by demonstrating that they can upregulate pol III transcription in these terminally differentiated cells.

Further work is required to establish the precise mechanisms by which ERK and c-Myc induce pol III transcription in cardiomyocytes. In proliferating cells, c-Myc interacts with TFIIIB, and is recruited to class III gene promoters (Gomez-Roman et al, 2003). This is presumed to underlie the activation of class III gene expression by c-Myc in such cells (Gomez-Roman et al, 2003). To determine whether this is also likely to explain the induction of pol III transcription by c-Myc in cardiomyocytes, co-immunoprecipitation analyses are required to demonstrate an interaction between endogenous c-Myc and TFIIIB. In addition, to examine the association of c-Myc with pol III promoters *in vivo*, a more sensitive assay for measuring c-Myc promoter occupancy needs to be developed, as ChIP experiments were unsuccessful in this regard. Assuming that c-Myc does activate the transcription of class III genes directly by binding to pol III-specific promoters, studies aimed at elucidating how this facilitates transcriptional activation will be of interest. c-Myc induces the transcription of pol II-transcribed target genes through the recruitment of coactivator proteins such as HATs (Bouchard et al, 2001; Frank et al, 2001; Frank et al, 2003). These enzymes modify histones to create a more open chromatin structure, which

facilitates access to the transcription machinery (Narlikar et al, 2002). Histone deacetylase (HDAC) enzymes antagonise the action of HATs, and maintain genes in an inactive state (Narlikar et al, 2002). HDACs have been shown to restrain class III gene expression in mammalian cells (Daly and White, unpublished results; Sutcliffe et al, 2000). Furthermore, HDACs play an important role in restricting the hypertrophic growth of cardiomyocytes (Chang et al, 2004; Zhang et al, 2002). Therefore, recruitment of HATs to class III genes by c-Myc would be expected to activate pol III transcription, and thus contribute to relieving the HDAC-mediated impairment of cardiomyocyte hypertrophy.

As demonstrated in Chapter 3 (Figure 3.8), extracts derived from hypertrophic cardiomyocytes transcribe pol III templates more actively *in vitro* than extracts from unstimulated cells. This suggests that, although chromatin structure may influence the rate of pol III transcription *in vivo*, hypertrophic stimulation also increases the inherent activity of the transcription machinery. To determine whether c-Myc directly participates in this chromatin-independent activation of pol III transcription, *in vitro* assays could be used. For example, if c-Myc activates pol III transcription solely by mediating changes in the chromatin environment, recombinant c-Myc would not be expected to activate pol III transcription *in vitro*. However, if c-Myc were to contribute to the direct activation of transcription by some other means, such as by promoting protein-protein interactions between components of the pol III transcription machinery, or by displacing the binding of a repressor protein, recombinant c-Myc would be predicted to augment pol III transcriptional activity in extracts derived from

unstimulated cells. Such experiments could provide insights into c-Myc-induced class III gene expression.

As with c-Myc, ERK interacts directly with the pol III transcription machinery in proliferating cells. Specifically, ERK binds and phosphorylates the Brf1 subunit of TFIIIB (Felton-Edkins et al, 2003a). This promotes transcription initiation by enhancing the interaction between TFIIIB and TFIIIC, and also TFIIIB and pol III, and is thought to account for the rapid induction of pol III transcription following the serum stimulation of fibroblasts (Felton-Edkins et al, 2003a). Although this is likely to contribute to the early activation of class III gene expression following hypertrophic stimulation (as depicted in Figure 7.2), phosphorylation of Brf1 by ERK remains to be formally demonstrated in cardiomyocytes. The possibility that ERK could induce pol III transcription by more indirect means was also considered in this study. For example, the involvement of ERK in the induction of the pol III-activator c-Myc was explored. This revealed that ERK can indeed induce c-Myc expression in these cells, which is likely to contribute to the ability of ERK to promote cardiomyocyte protein synthesis, not only through the activation of pol III transcription, but also via the induction of several other growth-promoting c-Myc targets identified in other cell types (Coller et al, 2000; Dang, 1999; Guo et al, 2000; O'Connell et al, 2003; Schmidt, 1999). In addition, ERK was shown to induce Brf1 expression, which, as discussed below, is also involved in the upregulation of pol III transcription during cardiomyocyte hypertrophy. Thus, these data are indicative of a central role for ERK in pol III transcriptional control in cardiomyocytes, as illustrated in Figure 7.1.

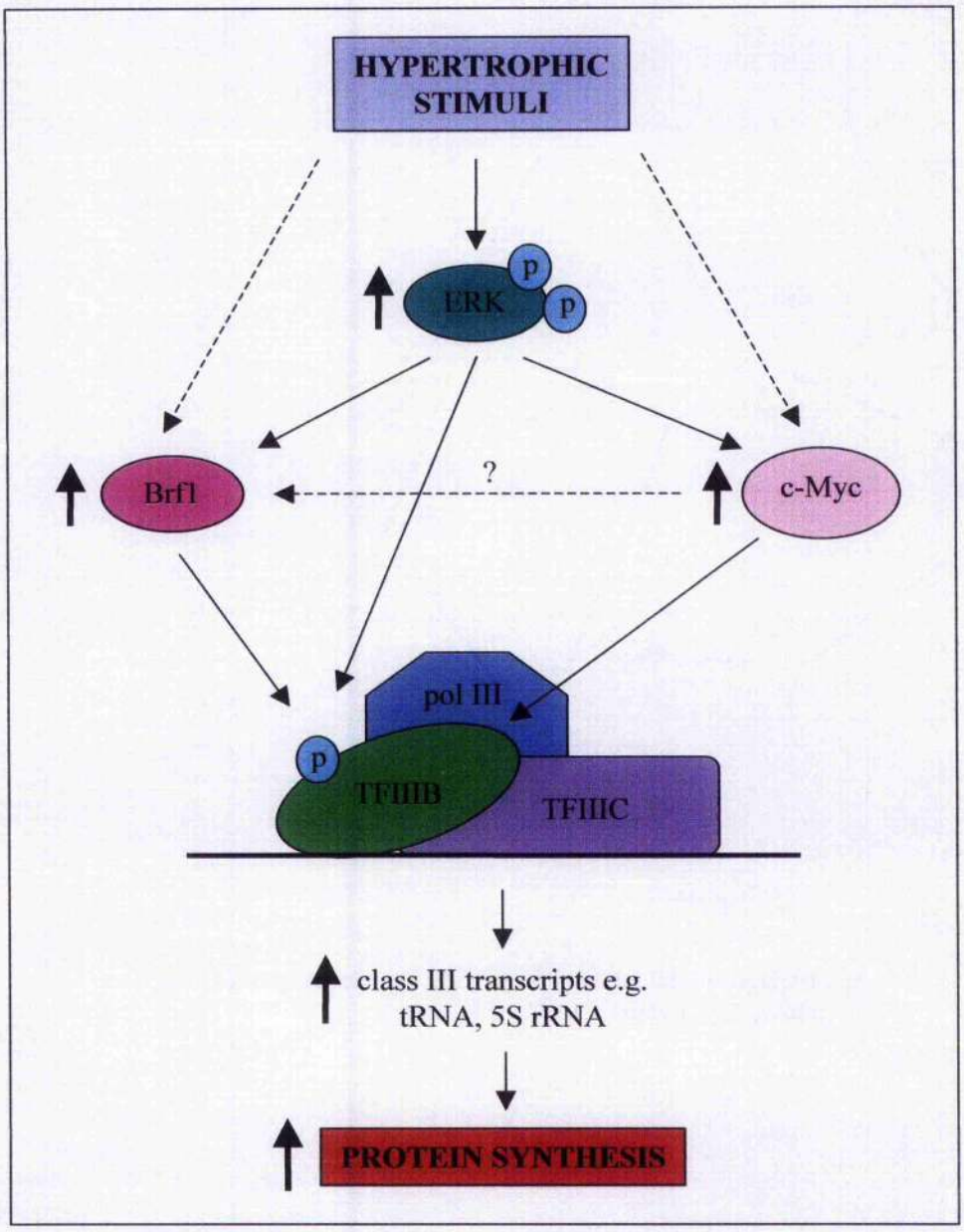


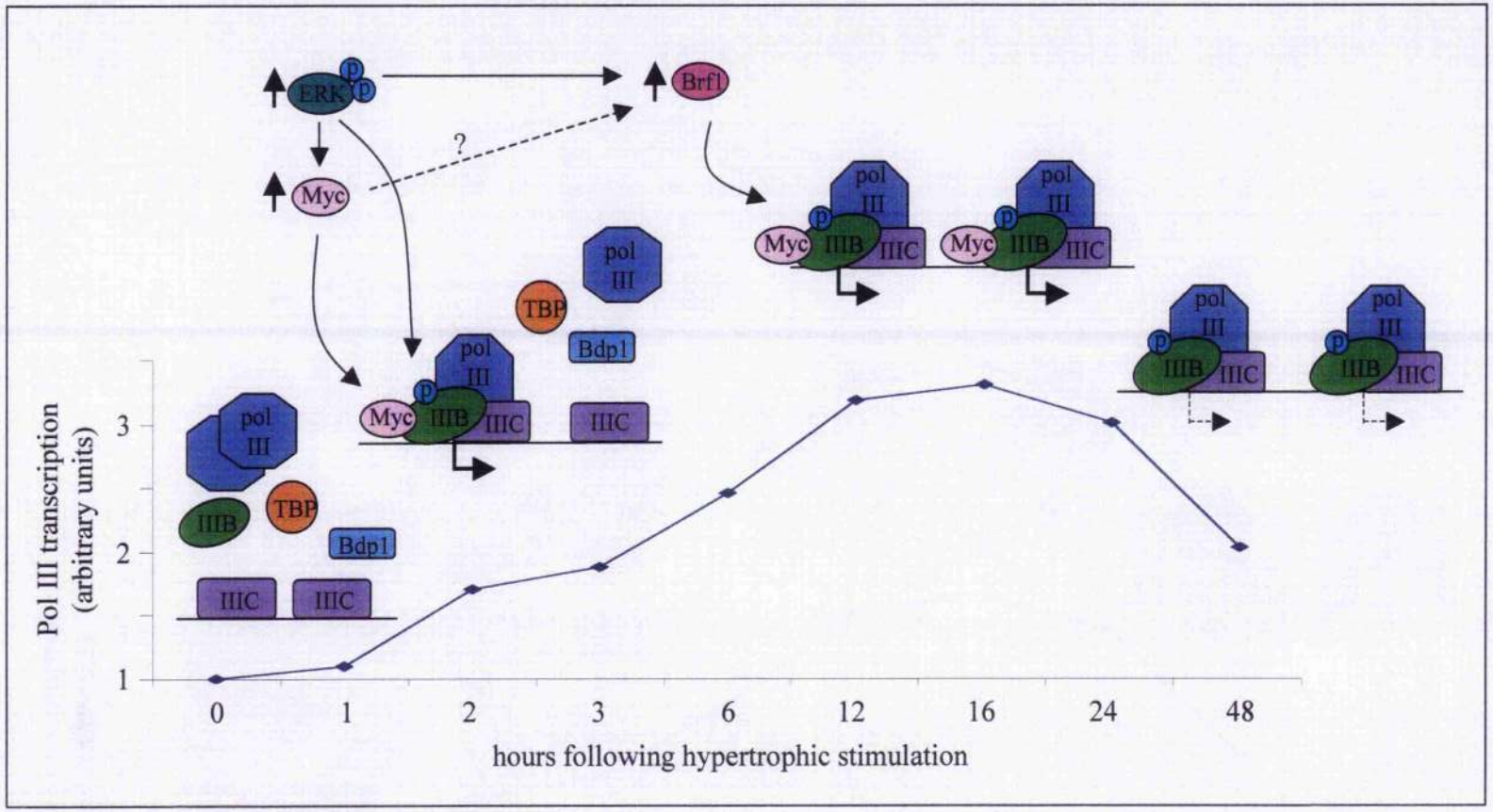
Figure 7.1: ERK is a central regulator of pol III transcription in cardiomyocytes. The various ways in which ERK is likely to induce pol III transcription in cardiomyocytes are illustrated in the diagram. These are as follows: 1. through the direct phosphorylation of TFIIIB; 2. through the induction of c-Myc; 3. through the activation of Brf1 expression. ERK-independent mechanisms also apparently contribute to c-Myc and Brf1 induction. Furthermore, c-Myc potentially regulates Brf1 expression via consensus E-box sequences found within or upstream of the Brf1 gene.

TFIIIB is composed of three subunits, Bdp1, Brf1 and TBP. Therefore, its activity will be influenced by the relative amounts of each of these components. In unstimulated cardiomyocytes, Brf1 levels are relatively low, and this was shown to limit pol III transcription. However, hypertrophic stimulation specifically increases the amount of Brf1, such that the abundance of this factor no longer restricts class III gene expression. Brf1 levels do not respond to the growth factor stimulation of fibroblasts (Figure 4.4A; Felton-Edkins et al, 2003a; Johnston et al, 2002; Scott et al, 2001). Therefore, this represents a novel mechanism for pol III transcriptional induction in mammalian cells, thus prompting an investigation into the mechanisms underlying this change in Brf1 abundance. Hypertrophic stimulation increases Brf1 at both the mRNA and protein level, consistent with enhanced transcription of the Brf1 gene. However, further work is required to confirm that elevated Brf1 transcripts do result from increased gene transcription, and not mRNA stability. As mentioned above, the induction of Brf1 in cardiomyocytes was shown to be at least partly mediated by ERK: ERK activation is sufficient to increase the level of Brf1 mRNA and protein, whereas ERK inhibition attenuates the induction of Brf1. This is the first study to report such ERK-controlled changes in Brf1 abundance. By inducing the expression of this limiting factor, ERK will indirectly activate pol III transcription. Analysis of the genomic region spanning the upstream end of the Brf1 gene suggested that c-Myc might also indirectly activate class III gene expression through the induction of Brf1 (as illustrated in Figures 7.1 and 7.2), as several potential c-Myc binding sites were identified. However, this has yet to be tested experimentally.

Further investigation is required to fully elucidate the mechanisms controlling the activation, or potentially the de-repression, of Brf1 expression during hypertrophic growth. Moreover, it will be important to establish whether growth factor responsive changes in Brf1 abundance are unique to cardiomyocytes, or a common feature of various different cell types. Conceivably, increased Brf1 expression may only be required to facilitate enhanced pol III transcription if basal Brf1 levels are relatively low, such as in differentiated cells (Alzuherri and White, 1998), or if demand for class III gene products is unusually high, for instance during tumour growth.

Thus, the hypertrophic stimulation of cardiomyocytes induces several molecular changes that can promote pol III transcription. It is likely that each of these changes, including ERK activation, c-Myc induction and increased Brf1 expression, will cooperate to attain maximal levels of class III gene expression, as illustrated in Figure 7.2. It is also probable that other pathways which promote hypertrophic growth will converge on the pol III transcription machinery in cardiomyocytes, in order to increase the capability for biosynthesis. For instance, signalling through PI3K has been implicated in the hypertrophic process, as the activation of this pathway is necessary and sufficient for the induction of cardiomyocyte protein synthesis and growth (Boluyt et al, 1997; Conderelli et al, 2002; Crackower et al, 2002; Schlüter et al, 1998; Schlüter et al, 1999; Shioi et al, 2000; Shioi et al, 2002). Consistent with the pol III transcription machinery being a common downstream target of growth-promoting signalling cascades, preliminary data indicate that PI3K regulates class III gene expression in cardiomyocytes: PI3K inhibition abrogates pol III transcription, whereas the

Figure 7.2: Mechanisms contributing to the induction of pol III transcription following the hypertrophic stimulation of cardiomyocytes. Hypertrophic stimulation leads to the rapid activation of ERK and c-Myc, which are likely to directly target TFIIIB, thus leading to the initial induction of pol III transcription. However, limiting levels of Brf1, and hence TFIIIB, potentially restrict the extent to which these molecules can activate the pol III transcription machinery at early timepoints. The increase in Brf1 abundance by 6 hours would relieve this restriction, thus allowing the attainment of maximum class III gene expression. Decreased c-Myc levels might account for the downregulation of pol III transcription occurring 24 to 48 hours after hypertrophic stimulation. Abbreviations used: TFIIIB, IIIB; TFIIIC, IIIC; c-Myc, Myc. Bold arrows below transcription complexes indicate active class III gene transcription; dashed arrows indicate transcription at a reduced rate.


ERK
P
P
Brf1
?
Myc
pol III
TBP
Bdp1
IIIB
IIIC
Pol III transcription (arbitrary units)
1
2
3
0
1
2
3
6
12
16
24
48
hours following hypertrophic stimulation

constitutive activation of this kinase in the heart is accompanied by elevated 5S rRNA levels (data not shown). Additional work is required to verify these findings, but they support the proposal that increased pol III transcription is critical for hypertrophic growth.

7.1.2 Pol III transcription is negatively regulated by RB in cardiomyocytes

RB is another example of a protein that interacts with TFIIIB in proliferating cells (Chu et al, 1997; Larminie et al, 1997). However, unlike ERK and c-Myc, this interaction facilitates pol III transcriptional repression (Chu et al, 1997; Larminie et al, 1997; Sutcliffe et al, 2000). Experiments carried out by WR MacLellan and colleagues (unpublished) have shown that RB is involved in restricting cardiomyocyte growth following hypertrophic stimulation of the heart. This finding challenges the traditionally held view that RB is principally concerned with regulating cell division, by demonstrating that cell growth inhibition may be an equally important, and in some cases primary, function of RB. Data presented in Chapter 5 provide an explanation for this RB-mediated growth restraint, by demonstrating that RB negatively regulates pol III transcription in these cells. This is the first study to show that RB controls pol III transcription *in situ* and in a non-dividing cell type. Thus, by inhibiting class III gene expression, RB could restrict cell growth, whether it be a prerequisite for cell division, as in the case of fibroblasts, or the hypertrophic enlargement of terminally differentiated cells.

In fibroblasts, hyperphosphorylation of RB, induced by mitogenic stimulation, relieves pol III transcriptional repression (Scott et al, 2001). Exposure of

cardiomyocytes to hypertrophic stimuli also induces RB phosphorylation (Figures 4.3 and 4.6; Nozato et al, 2001; Sadoshima et al, 1997). However, although RB is clearly involved in limiting the rate of pol III transcription under hypertrophic conditions in the heart, the relevance of RB phosphorylation to the induction of class III gene expression during hypertrophy has yet to be established. Adenoviral-mediated expression of a non-phosphorylatable RB mutant in cultured cardiomyocytes could be used to investigate this possibility.

7.2 Control of U6 snRNA and 7SK gene expression

The mechanisms described above are likely to regulate the expression of the majority of class III genes, by targeting a common component of the basal transcription machinery, namely TFIIIB, and thus will impact on the production of numerous RNAs involved in cellular metabolism. However, a small number of pol III-transcribed genes utilise a distinct TFIIIB complex. Specifically, those with a type 3 promoter, including the U6 snRNA and 7SK genes, do not require Brf1, but instead utilise the related factor Brf2 (Schramm and Hernandez, 2002; Schramm et al, 2000). Furthermore, this TFIIIB-like complex is recruited to type 3 promoters through its interactions with $SNAP_c$, rather than with TFIIIC (Schramm and Hernandez, 2002). In fact, TFIIIC is not involved in the expression of these genes at all (see Chapter 1). It would be interesting to determine whether the transcription of such genes is regulated by the same mechanisms that control transcription from type 1 and 2 promoters, or whether entirely different mechanisms are employed. Potentially, Brf2 may substitute for Brf1 as a target for regulatory strategies, with the abundance and phosphorylation

status of this subunit determining the rate of production of such transcripts. Alternatively, $SNAP_c$ may be an additional target of known pol III regulators, allowing the coordinate induction of all types of class III gene.

7.3 Potential for the coordinated production of components of the translational apparatus during cardiomyocyte hypertrophy

Pol III transcription is often coordinated with transcription by pol I, thus allowing the simultaneous production of essential components of the cellular biosynthetic machinery. In keeping with this, class I gene expression increases in response to the hypertrophic stimulation of cardiomyocytes (Hannan et al, 1995; Hannan et al, 1996a; Luyken et al, 1996; McDermott et al, 1989; McDermott et al, 1991). It is probable that this synchronous induction of class I and III gene transcription is achieved by the use of common regulatory mechanisms. Studies on the control of pol I transcription in cardiomyocytes have suggested that an increase in the phosphorylation and abundance of the pol I-specific transcription factor UBF regulates the activation of class I gene expression during hypertrophy (Brandenburger et al, 2001; Brandenburger et al, 2003; Hannan et al, 1995; Hannan et al, 1996a, b; Luyken et al, 1996). Although it has not yet been determined which signal transduction pathways are responsible for this control, ERK, c-Myc and RB are likely to be involved. For example, ERK-directed phosphorylation of UBF has been documented to mediate growth factor-stimulated increases in pol I transcription in other cell types (Stefanovsky et al,

2001). Furthermore, UBF expression is controlled by c-Myc in differentiating granulocytes (Poortinga et al, 2004). In addition, RB interacts with UBF leading to pol I transcriptional repression in proliferating cells (Hannan et al, 2000). Thus, by targeting TFIIIB and UBF, these regulatory mechanisms could effectively coordinate class I and III gene expression in order to meet the biosynthetic needs of growing cardiomyocytes.

The activation of pol I transcription is essential for cardiomyocyte growth (Brandenburger et al, 2001). Given that pol III transcribes complementary components of the protein synthetic machinery, and that numerous regulatory mechanisms are involved in ensuring the appropriate expression of class III genes, it is highly probable that increased pol III transcription will also be a critical feature of cardiomyocyte hypertrophy, and indeed cell growth in general. Ultimately, further investigation is required to confirm this likelihood, by specifically preventing the hypertrophy-associated increase in pol III transcription, and analysing the effects of this inhibition on cell growth. Such exploration depends on the availability of agents that will specifically inhibit the expression of class III genes. This could be achieved by various means. For example, given that Brf1 is essential for tRNA and 5S rRNA production, antisense mRNA or siRNA directed against this factor could be used to knock down Brf1 during hypertrophy, and thus specifically diminish the expression of these and many other pol III-transcribed genes to basal levels. Furthermore, a recent study has demonstrated that the bacterial compound tagetitoxin, which had previously been shown to specifically abrogate pol III transcription *in vitro* and in *Xenopus laevis* oocytes (Steinberg et al, 1990), can also be used to inhibit pol

III transcription in cultured mammalian cells (Allen et al, 2004). These approaches are likely to yield valuable information regarding the mechanisms underlying cell growth, with particular relevance to a process associated with heart disease.

In summary, this thesis describes an unprecedented investigation into the growth-associated regulation of pol III transcription in a non-dividing, terminally differentiated cell type. Moreover, the work presented contributes to the current understanding of the mechanisms underlying cardiomyocyte hypertrophy, by demonstrating that class III gene expression is subject to control during hypertrophic growth through the concerted action of several proteins. Appreciating the molecular basis of hypertrophic growth has important implications for the rational design of therapies aimed at treating and preventing sustained myocardial hypertrophy, and its associated human pathologies.

References

Akazawa, H. and Komuro, I. Roles of cardiac transcription factors in cardiac hypertrophy. *Circulation Research.* 2003; **92**:1079-1088.

Akhter, S.A., Luttrell, L.M., Rockman, H.A., Iaccarino, G., Lefkowitz, R.J., and Koch, W.J. Targeting the receptor-G_q interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science.* 1998; **280**:574-577.

Akusjärvi, G., Mathews, M.B., Andersson, P., Vennstrom, B., and Pettersson, U. Structure of genes for virus associated RNA_I and RNA_{II} of adenovirus type 2. *Proc.Natl.Acad.Sci.USA.* 1980; **77**:2424-2428.

Allen, T.A., Von Kaenel, S., Goodrich, J.A., and Kugel, J.F. The SINE-encoded mouse B2 RNA represses mRNA transcription in response to heat shock. *Nature Structural & Molecular Biology.* 2004; **11**:816-821.

Alzuherri, H.M. and White, R.J. Regulation of a TATA-binding protein-associated factor during cellular differentiation. *Journal of Biological Chemistry.* 1998; **273**:17166-17171.

Amati, B., Frank, S.R., Donjerkovic, D., and Taubert, S. Function of the c-Myc oncoprotein in chromatin remodeling and transcription. *Biochimica et Biophysica Acta.* 2001; **1471**:M135-M145.

Antos, C.L., McKinsey, T.A., Frey, N., Kutschke, W., McAnally, J., Shelton, J.M., Richardson, J.A., Hill, J.A., and Olson, E.N. Activated glycogen synthase kinase-3β suppresses cardiac hypertrophy *in vivo*. *Proc.Natl.Acad.Sci.USA.* 2002; **99**:907-912.

Anversa, P. and Kajstura, J. Ventricular myocytes are not terminally differentiated in the adult mammalian heart. *Circulation Research.* 1998; **83**:1-14.

Anversa, P. and Nadal-Ginard, B. Myocyte renewal and ventricular remodelling. *Nature.* 2002; **415**:240-243.

Babu, G.L., Lalli, M.J., Sussman, M.A., Sadoshima, J., and Periasamy, M. Phosphorylation of elk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy. *Journal of Molecular and Cellular Cardiology*. 2000; **32**:1447-1457.

Baker, R.E., Gabrielsen, O., and Hall, B.D. Effects of $tRNA^{Tyr}$ point mutations on the binding of yeast RNA polymerase III transcription factor C. *Journal of Biological Chemistry*. 1986; **261**:5275-5282.

Bartholomew, B., Kassavetis, G.A., and Geiduschek, E.P. The subunit structure of *Saccharomyces cerevisiae* transcription factor IIIC probed with a novel photocrosslinking reagent. *EMBO Journal*. 1990; **9**:2197-2205.

Bartkiewicz, M., Gold, H., and Altman, S. Identification and characterization of an RNA molecule that copurifies with RNase P activity in HeLa cells. *Genes and Development*. 1989; **3**:488-499.

Baxter, G.C. and Stanners, C.P. The effect of protein degradation on cellular growth characteristics. *Journal of Cellular Physiology*. 1978; **96**:139-146.

Beltrami, A.P., Urbanek, K., Kajstura, J., Yan, S.M., Finato, N., Bussani, R., Nadal-Ginard, B., Silvestri, F., Leri, A., Beltrami, A., and Anversa, P. Evidence that human cardiac myocytes divide after myocardial infarction. *New England Journal of Medicine*. 2001; **344**:1750-1757.

Bhat, R.A. and Thimmappaya, B. Construction and analysis of additional adenovirus substitution mutants confirm the complementation of VA_I RNA function by two small RNAs encoded by Epstein-Barr virus. *Journal of Virology*. 1985; **56**:750-756.

Bishopric, N.H., Simpson, P.C., and Ordahl, C.P. Induction of skeletal α-actin gene in α_1-adrenoceptor-mediated hypertrophy of rat cardiac myocytes. *Journal of Clinical Investigation*. 1987; **80**:1194-1199.

Bishopric, N.H., Jayasena, V., and Webster, K.A. Positive regulation of the skeletal α-actin gene by Fos and Jun in cardiac myocytes. *Journal of Biological Chemistry.* 1992; **267**:25535-25540.

Bladon, T.S., Frégeau, C.J., and McBurney, M.W. Synthesis and processing of small B2 transcripts in mouse embryonal carcinoma cells. *Molecular and Cellular Biology.* 1990; **10**:4058-4067.

Bogenhagen, D.F. The intragenic control region of the *Xenopus* 5S gene contains two factor A binding domains that must be aligned properly for efficient transcription initiation. *Journal of Biological Chemistry.* 1985; **260**:6466-6471.

Bogoyevitch, M.A., Glennon, P.E., and Sugden, P. Endothelin-1, phorbol esters and phenylephrine stimulate MAP kinase activities in ventricular cardiomyocytes. *FEBS Letters.* 1993; **317**:271-275.

Bogoyevitch, M.A., Glennon, P.E., Andersson, M.B., Clerk, A., Lazou, A., Marshall, C.J., Parker, P.J., and Sugden, P. Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. *Journal of Biological Chemistry.* 1994; **269**:1110-1119.

Boluyt, M.O., Zheng, J.-S., Younes, A., Long, X., O'Neill, L., Silverman, H., Lakatta, E.G., and Crow, M.T. Rapamycin inhibits α_1-adrenergic receptor-stimulated cardiac myocyte hypertrophy but not activation of hypertrophy-associated genes. *Circulation Research.* 1997; **81**:176-186.

Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorage, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO Journal.* 1999; **18**:5321-5333.

Bouchard, C., Dittrich, O., Kiermaier, A., Dohmann, K., Menkel, A., Eilers, M., and Lüscher, B. Regulation of cyclin D2 gene expression by the Myc/Max/Mad network:

Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. *Genes and Development*. 2001; **15**:2042-2047.

Brandenburger, Y., Jenkins, A., Autelitano, D.J., and Hannan, R.D. Increased expression of UBF is a critical determinant of rRNA synthesis and hypertrophic growth of cardiac myocytes. *FASEB Journal*. 2001; **15**:2051-2053.

Brandenburger, Y., Arthur, J.F., Woodcock, E.A., Du, X.J., Gao, X.M., Autelitano, D.J., Rothblum, L.I., and Hannan, R.D. Cardiac hypertrophy in vivo is associated with increased expression of the ribosomal gene transcription factor UBF. *FEBS Letters*. 2003; **548**:79-84.

Braz, J.C., Bueno, O.F., De Windt, L.J., and Molkentin, J.D. PKCα regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase 1/2 (ERK1/2). *Journal of Cell Biology*. 2002; **156**:905-919.

Brooks, G., Poolman, R.A., and Li, J.M. Arresting developments in the cardiac myocyte cell cycle: Role of cyclin-dependent kinase inhibitors. *Cardiovascular Research*. 1998; **39**:301-311.

Brown, D.D., Wensink, P.C., and Jordan, E. Purification and some characteristics of 5S DNA from *Xenopus laevis*. *Proc.Natl.Acad.Sci.USA*. 1971; **68**:3175-3179.

Brun, I., Sentenac, A., and Werner, M. Dual role of the C34 subunit of RNA polymerase III in transcription initiation. *EMBO Journal*. 1997; **16**:5730-5741.

Bueno, O.F., De Windt, L.J., Tymitz, K.M., Witt, S.A., Kimball, T.R., Klevitsky, R., Hewett, T.E., Jones, S.P., Lefer, D.J., Peng, C., Kitis, R.N., and Molkentin, J.D. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO Journal*. 2000; **19**:6341-6350.

Bueno, O.F. and Molkentin, J.D. Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. *Circulation Research*. 2002; **91**:776-781.

Bugaisky, L.B., Gupta, M., Gupta, M.P., and Zak, R. Cellular and molecular mechanisms of cardiac hypertrophy. *In The heart and cardiovascular system, second edition.* Editors: Fozzard, H.A., Haber, E., Jennings, R.B., Katz, A.M. and Morgan, H.E. Raven Press, New York; 1992: 1621-1656.

Busk, P.K., Bartkova, J., Strøm, C.C., Wulf-Andersen, L., Hinrichsen, R., Christoffersen, T.E.H., Latella, L., Bartek, J., Haunsø, S., and Sheikh, S.P. Involvement of cyclin D in left ventricular hypertrophy in vivo and in vitro. *Cardiovascular Research.* 2002; **56**:64-75.

Cairns, C.A. and White, R.J. p53 is a general repressor of RNA polymerase III transcription. *EMBO Journal.* 1998; **17**:3112-3123.

Camacho, J.A., Peterson, C.J., White, G.J., and Morgan, H.E. Accelerated ribosome formation and growth in neonatal pig hearts. *American Journal of Physiology.* 1990; **258**:C86-C91.

Cavanaugh, A.H., Hempel, W.M., Taylor, L.J., Rogalsky, V., Todorov, G., and Rothblum, L.I. Activity of RNA polymerase I transcription factor UBF blocked by *Rb* gene product. *Nature.* 1995; **374**:177-180.

Chakrabarti, O., Veeraraghavalu, K., Tergaonkar, V., Liu, Y., Androphy, E.J., Stanley, M.A., and Krishna, S. Human papillomavirus type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signalling and differentially regulate tumorigenesis by notch signalling and oncogenic ras. *Journal of Virology.* 2004; **78**:5934-5945.

Chang, F., Steelman, L.S., Shelton, J.G., Lee, J.T., Navolanic, P.M., Blalock, W.L., Franklin, R., and McCubrey, J.A. Regulation of cell cycle progression and apoptosis by the ras/raf/MEK/ERK pathway. *International Journal of Oncology.* 2003; **22**:469-480.

Chang, L. and Karin, M. Mammalian MAP kinase signalling cascades. *Nature.* 2001; **410**:37-40.

Chang, S., McKinsey, T.A., Zhang, C.L., Richardson, J.A., Hill, J.A., and Olson, E.N. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals ad play redundant roles in heart development. *Molecular and Cellular Biology*. 2004; **24**:8467-8476.

Cheng, M., Wang, D., and Roussel, M.F. Expression of c-Myc in response to colony-stimulating factor-1 requires mitogen-activated protein kinase kinase 1. *Journal of Biological Chemistry*. 1999; **274**:6553-6558.

Chien, K.R., Knowlton, K.U., Zhu, H., and Chien, S. Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiological response. *FASEB Journal*. 1991; **5**:3037-3046.

Chu, W.M., Wang, Z., Roeder, R.G., and Schmid, C.W. RNA polymerase III transcription repressed by RB through its interactions with TFIIIB and TFIIIC2. *Journal of Biological Chemistry*. 1997; **272**:14755-14761.

Chu, W.M., Ballard, R., Caprick, B.W., Williams, B.R.G., and Schmid, C.W. Potential Alu function: regulation of the activity of double-stranded RNA-activated kinase PKR. *Molecular and Cellular Biology*. 1998; **18**:58-68.

Chua, B.H.L., Russo, L.A., Gordon, E.E., Kleinhans, B.J., and Morgan, H.E. Faster ribosome synthesis induced by elevated aortic pressure in rat heart. *American Journal of Physiology*. 1987; **252**:C323-C327.

Ciechanover, A. The ubquitin-proteasome pathway: on protein death and cell life. *EMBO Journal*. 1998; **17**:7151-7160.

Ciliberto, G., Raugei, S., Constanzo, F., Dente, L., and Cortese, R. Common and interchangeable elements in the promoters of genes transcribed by RNA polymerase III. *Cell*. 1983; **32**:725-733.

Clarke, E.M., Peterson, C.L., Brainard, A.V., and Riggs, D.L. Regulation of the RNA polymerase I and III transcription systems in response to growth conditions. *Journal of Biological Chemistry*. 1996; **271**:22189-22195.

Clerk, A., Bogoyevitch, M.A., Andersson, M.B., and Sugden, P. Differential activation of protein kinase C isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 mitogen-activated protein kinases in ventricular myocytes cultures from neonatal rat hearts. *Journal of Biological Chemistry.* 1994; **269**:32848-32857.

Clerk, A. and Sugden, P.H. Activation of protein kinase cascades in the heart by hypertrophic G protein-coupled receptor agonists. *American Journal of Cardiology.* 1999; **83**:64H-69H.

Coller, H.A., Grandori, C., Tamayo, P., Colbert, T., Lander, E.S., Eisenman, R.N., and Golub, T.R. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc.Natl.Acad.Sci.USA.* 2000; **97**:3260-3265.

Condorelli, G., Drusco, A., Stassi, G., Bellacosa, G., Roncarati, R., Iaccarino, G., Russo, M.A., Gu, Y., Dalton, N., Chung, C., Latronico, M.V.G., Napoli, C., Sadoshima, J., Croce, C.M., and Ross, J.Jr. Akt induces enhanced myocardial contractility and cell size *in vivo* in transgenic mice. *Proc.Natl.Acad.Sci.USA.* 2002; **99**:12333-12338.

Conlon, I. and Raff, M. Size control in animal development. *Cell.* 1999; **96**:235-244.

Connell-Crowley, L., Harper, J.W., and Goodrich, D.W. Cyclin D1/cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. *Molecular Biology of the Cell.* 1997; **8**:287-301.

Cormack, B.P. and Struhl, K. The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. *Cell.* 1992; **69**:685-696.

Crackower, M.A., Oudit, G.Y., Kozieradzki, I., Sarao, R., Sun, H., Sasaki, T., Hirsch, E., Suzuki, A., Shioi, T., Irie-Sasaki, J., Sah, R., Cheng, H.Y.M., Rybin,

V.O., Lembo, G., Fratta, L., Oliveira-dos-Santos, A.J., Benovic, J.L., Kahn, C.R., Izumo, S., Steinberg, S.F., Wymann, M.P., Backx, P.H., and Penninger, J.M. Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell.* 2002; **110**:737-749.

Creighton, T.E. *Proteins, second edition.* W.H.Freeman and Company. New York. 1997.

Crighton, D., Woiwode, A., Zhang, C., Mandavia, N., Morton, J.P., Warnock, L.J., Milner, J., White, R.J., and Johnson, D.L. p53 represses RNA polymerase III transcription by targeting TBP and inhibiting promoter occupancy by TFIIIB. *EMBO Journal.* 2003; **22**:2810-2820.

Cutilletta, A.F., Rudnik, M., and Zak, R. Muscle and non-muscle cell RNA polymerase activity during the development of myocardial hypertrophy. *Journal of Molecular and Cellular Cardiology.* 1978; **10**:677-687.

D'Angelo, D.D., Sakata, Y., Lorenz, J.N., Boivin, G.P., Walsh, R.A., Liggett, S.B., and Dorn II, G.W. Transgenic Gαq overexpression induces cardiac contractile failure in mice. *Proc.Natl.Acad.Sci.USA.* 1997; **94**:8121-8126.

Daly, N.L., Arvanitis, D.A., Fairley, J.A., Gomez-Roman, N., Morton, J.P., Grahem, S.V., Spandidos, D.A., and White, R.J. *Oncogene.* 2004; **In press.**

Dang, C.V. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Molecular and Cellular Biology.* 1999; **19**:1-11.

Das, G., Henning, D., Wright, D., and Reddy, R. Upstream regulatory elements are necessary and sufficient for transcription of a U6 RNA gene by RNA polymerase III. *EMBO Journal.* 1988; **7**:503-512.

Datar, S.A., Jacobs, H.W., de la Cruz, A.F.A., Lehner, C.F., and Edgar, B.A. The *Drosophila* cyclin D-cdk4 complex promotes cellular growth. *EMBO Journal.* 2000; **19**:4543-4554.

Dean, M., Levine, R.A., and Campisi, J. c-myc regulation during retinoic acid-induced differentiation of F9 cells is posttranscriptional and associated with growth arrest. *Molecular and Cellular Biology.* 1986; **6**:518-524.

Dieci, G. and Sentenac, A. Facilitated recycling pathway for RNA polymerase III. *Cell.* 1996; **84**:245-252.

Diehl, J.A., Zindy, F., and Sherr, C.J. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes and Development.* 1997; **11**:957-972.

Dorn II, G.W. and Brown, J.H. Gq signaling in cardiac adaptation and maladaptation. *Trends in Cardiovascular Medicine.* 1999; **9**:26-34.

Doudna, J.A. and Rath, V.L. Structure and function of the eukaryotic ribosome: the next frontier. *Cell.* 2002; **109**:153-156.

Dyson, N. The regulation of E2F by pRB-family proteins. *Genes and Development.* 1998; **12**:2245-2262.

Eichhorn, K. and Jackson, S.P. A role for TAF3B2 in the repression of human RNA polymerase III transcription in nonproliferatng cells. *Journal of Biological Chemistry.* 2001; **276**:21158-21165.

Elion, E.A. and Warner, J.R. The major promoter element of rRNA transcription in yeast lies 2 kb upstream. *Cell.* 1984; **39**:663-673.

Engelke, D.R., Ng, S.-Y., Shastry, B.S., and Roeder, R.G. Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. *Cell.* 1980; **19**:717-728.

Espinoza, C.A., Allen, T.A., Hieb, A.R., Kugel, J.F., and Goodrich, J.A. B2 RNA binds directly to RNA polymerase II to repress transcript synthesis. *Nature Structural & Molecular Biology.* 2004; **11**:822-829.

Everett, A.D., Stoops, T.D., Nairn, A.C., and Brautigan, D. Angiotensin II regulates phosphorylation of translation elongation factor-2 in cardiac myocytes. *American Journal of Physiology*. 2001; **281**:H161-H167.

Fabrizio, P., Coppo, A., Fruscoloni, P., Benedetti, P., Di Segni, G., and Tocchini-Valentini, G.P. Comparative mutational analysis of wild-type and stretched $tRNA^{Leu3}$ gene promoters. *Proc.Natl.Acad.Sci.USA*. 1987; **84**:8763-8767.

Fairley, J.A., Scott, P.H., and White, R.J. TFIIIB is phosphorylated, disrupted and selectively released from tRNA promoters during mitosis *in vivo*. *EMBO Journal*. 2003; **22**:5841-5850.

Felton-Edkins, Z.A. and White, R.J. Multiple mechanisms contribute to the activation of RNA polymerase III transcription in cells transformed by papovaviruses. *Journal of Biological Chemistry*. 2002; **277**:48182-48191.

Felton-Edkins, Z.A., Fairley, J.A., Graham, E.L., Johnston, I.M., White, R.J., and Scott, P.H. The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIIB. *EMBO Journal*. 2003a; **22**:2422-2432.

Felton-Edkins, Z.A., Kenneth, N.S., Brown, T.R.P., Daly, N.L., Gomez-Roman, N., Grandori, C., Eisenman, R.N., and White, R.J. Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc. *Cell Cycle*. 2003b; **2**:181-184.

Ferrari, R., Rivetti, C., Acker, J., and Dieci, G. Distinct roles of transcription factors TFIIIB and TFIIIC in RNA polymerase III transcription reinitiation. *Proc.Natl.Acad.Sci.USA*. 2004; **101**:13442-13447.

Fingar, D.C., Salama, S., Tsou, C., Harlow, E., and Blenis, J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes and Development*. 2002; **16**:1472-1487.

Flesch, M., Margulies, K.B., Mochmann, H.C., Engel, D., Sivasubramanian, N., and Mann, D.L. Differential regulation of mitogen-activated protein kinases in the

failing human heart in response to mechanical unloading. *Circulation*. 2001; **104**:2273-2276.

Flink, I.L., Oana, S., Maitra, N., Bahl, J.J., and Morkin, E. Changes in E2F complexes containing retinoblastoma protein family members and increased cyclin-dependent kinase inhibitor activities during terminal differentiation of cardiomyocytes. *Journal of Molecular and Cellular Cardiology*. 1998; **30**:563-578.

Flores, A., Briand, J.F., Gadal, O., Andrau, J.C., Rubbi, L., Van Mullem, V., Boschiero, C., Goussot, M., Marck, C., Carles, C., Thuriaux, P., Sentenac, A., and Werner, M. A protein-protein interaction map of yeast RNA polymerase III. *Proc.Natl.Acad.Sci.USA*. 1999; **96**:7815-7820.

Francis, M.A. and Rajbhandary, U.L. Expression and function of a human initiator tRNA gene in the yeast *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*. 1990; **10**:4486-4494.

Frank, S.R., Schroeder, M., Fernandez, P., Taubert, S., and Amati, B. Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes and Development*. 2001; **15**:2069-2082.

Frank, S.R., Parisi, T., Taubert, S., Fernandez, P., Fuchs, M., Chan, H.M., Livingston, D.M., and Amati, B. MYC recruits the TIP60 histone acetyltransferase complex to chromatin. *EMBO Reports*. 2003; **4**:575-580.

Frey, N. and Olson, E.N. Cardiac hypertrophy: the good, the bad and the ugly. *Annual Reviews of Physiology*. 2003; **65**:21.1-21.35.

Galli, G., Hofstetter, H., and Birnstiel, M.L. Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements. *Nature*. 1981; **294**:626-631.

Garber, M., Panchanathan, S., Fan, R.S., and Johnson, D.L. The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, induces specific transcription by RNA polymerase III in Drosophila Schneider cells. *Journal of Biological Chemistry*. 1991; **266**:20598-20601.

Garrington, T.P. and Johnson, G.L. Organization and regulation of mitogen-activate protein kinase signaling pathways. *Current Opinion in Cell Biology*. 1999; **11**:211-218.

Geiduschek, E.P. and Kassavetis, G.A. The RNA polymerase III transcription apparatus. *Journal of Molecular Biology*. 2001; **310**:1-26.

Ghavidel, A. and Schultz, M.C. Casein kinase II regulation of yeast TFIIIB is mediated by the TATA binding protein. *Genes and Development*. 1997; **11**:2780-2789.

Ghavidel, A. and Schultz, M.C. TATA binding protein-associated CK2 transduces DNA damage signals to the RNA polymerase III transcriptional machinery. *Cell*. 2001; **106**:575-584.

Gillespie-Brown, J., Fuller, S.J., Bogoyevitch, M.A., Cowley, S., and Sugden, P.H. The mitogen-activated protein kinase kinase MEK1 stimulates a pattern of gene expression typical of the hypertrophic phenotype in rat ventricular cardiomyocytes. *Journal of Biological Chemistry*. 1995; **270**:28092-28096.

Ginsberg, A.M., King, B.O., and Roeder, R.G. *Xenopus* 5S gene transcription factor, TFIIIA:characterization of a cDNA clone and measurement of RNA levels throughout development. *Cell*. 1984; **39**:479-489.

Glennon, P.E., Kaddoura, S., Sale, E.M., Sale, G.J., Fuller, S.J., and Sugden, P. Depletion of mitogen-activated protein kinase using an antisense oligodeoxynucleotide approach downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes. *Circulation Research*. 1996; **78**:954-961.

Gokal, P.K., Cavanaugh, A.H., and Thompson, E.A. The effects of cyclohexamide upon transcription of rRNA, 5S RNA, and tRNA genes. *Journal of Biological Chemistry*. 1986; **261**:2536-2541.

Gomez-Roman, N., Grandori, C., Eisenman, R.N., and White, R.J. Direct activation of RNA polymerase III transcription by c-Myc. *Nature*. 2003; **421**:290-294.

Gottesfeld, J.M., Wolf, V.J., Dang, T., Forbes, D.J., and Hartl, P. Mitotic repression of RNA polymerase III transcription in vitro mediated by phosphorylation of a TFIIIB component. *Science*. 1994; **263**:81-84.

Graña, X., Garriga, J., and Mayol, X. Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene*. 1998; **17**:3365-3383.

Grandori, C., Cowley, S.M., James, L.P., and Eisenman, R.N. The Myc/Max/Mad network and the transcriptional control of cell behaviour. *Annual Review of Cell and Developmental Biology*. 2000; **16**:653-699.

Greasley, P.J., Bonnard, C., and Amati, B. Myc induces the *nucleolin* and *BN51* genes: possible implications in ribosome biogenesis. *Nucleic Acids Research*. 2000; **28**:446-453.

Grummt, I. Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes and Development*. 2003; **17**:1691-1702.

Gu, Z. and Matlashewski, G. Effect of human papillomavirus type 16 oncogenes on MAP kinase activity. *Journal of Virology*. 1995; **69**:8051-8056.

Guo, Q.M., Malek, R.L., Kim, S., Chiao, C., He, M., Ruffy, M., Sanka, K., Lee, N.H., Dang, C.V., and Liu, E.T. Identification of c-Myc responsive genes using rat cDNA microarray. *Cancer Research*. 2000; **60**:5922-5928.

Gusterson, R., Brar, B., Faulkes, D., Giordano, A., Chrivia, J., and Latchman, D. The transcriptional co-activators CBP and p300 are activated via phenylephrine through the p42/p44 MAPK cascade. *Journal of Biological Chemistry*. 2002; **277**:2517-2524.

Hannan, K.M., Kennedy, B.K., Cavanaugh, A.H., Hannan, R.D., Hirschler-Laszkiewicz, I., Jefferson, L.S., and Rothblum, L.I. RNA polymerase I transcription in confluent cells: Rb downregulates rDNA transcription during confluence-induced cell cycle arrest. *Oncogene*. 2000; **19**:3487-3497.

Hannan, R.D., Luyken, J., and Rothblum, L.I. Regulation of rDNA transcription factors during cardiomyocyte hypertrophy induced by adrenergic agents. *Journal of Biological Chemistry*. 1995; **270**:8290-8297.

Hannan, R.D. and Rothblum, L.I. Regulation of ribosomal DNA transcription during neonatal cardiomyocyte hypertrophy. *Cardiovascular Research*. 1995; **30**:501-510.

Hannan, R.D., Luyken, J., and Rothblum, L.I. Regulation of ribosomal DNA transcription during contraction-induced hypertrophy of neonatal cardiomyocytes. *Journal of Biological Chemistry*. 1996a; **271**:3213-3220.

Hannan, R.D., Stefanovsky, V., Taylor, L., Moss, T., and Rothblum, L.I. Overexpression of the transcription factor UBF1 is sufficient to increase ribosomal DNA transcription in neonatal cardiomyocytes: Implications for cardiac hypertrophy. *Proc.Natl.Acad.Sci.USA*. 1996b; **93**:8750-8755.

Hannan, R.D., Jenkins, A., Jenkins, A.K., and Brandenburger, Y. Cardiac hypertrophy: a matter of translation. *Clinical and Experimental Pharmacology and Physiology*. 2003; **30**:517-527.

Haq, S., Michael, A., Andreucci, M., Bhattacharya, K., Dotto, P., Walters, B., Woodgettt, J., Kilter, H., and Force, T. Stabilization of β-catenin by a Wnt-independent mechanism regulates cardiomyocyte growth. *Proc.Natl.Acad.Sci.USA*. 2003; **100**:4610-4615.

Harbour, J.W., Luo, R.X., Dei Santi, A., Postigo, A.A., and Dean, D.C. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*. 1999; **98**:859-869.

Hardt, S.E., Tomita, H., Katus, H.A., and Sadoshima, J. Phosphorylation of eukaryotic translation initiation factor 2Bε by glycogen synthase kinase-3β regulates β-adrenergic cardiac myocyte hypertrophy. *Circulation Research.* 2004; **94**:926-935.

Harismendy, O., Gendrel, C.G., Soularue, P., Gidrol, X., Sentenac, A., Werner, M., and Lefebvre, O. Genome-wide location of yeast RNA polymerase III transcription machinery. *EMBO Journal.* 2003; **22**:4738-4747.

Harjai, K.J. Potential new cardiovascular risk factors: left ventricular hypertrophy, homocysteine, lipoprotein(a), triglycerides, oxidative stress and fibrinogen. *Annals of Internal Medicine.* 1999; **131**:376-386.

Harris, I.S., Zhang, S., Treskov, I., Kovacs, A., Weinheimer, C., and Muslin, A.J. Raf-1 kinase is required for cardiac hypertrophy and cardiomyocyte survivial in response to pressure overload. *Circulation.* 2004; **110**:718-723.

Hastings, M.L. and Krainer, A.R. Pre-mRNA splicing in the new millennium. *Current Opinion in Cell Biology.* 2001; **13**:302-309.

Herzig, T.C., Jobe, S.M., Aoki, H., Molkentin, J.D., Cowley, J.R., Izumo, S., and Markham, B.E. Angiotensin II type 1a receptor gene expression in the heart: AP-1 and GATA-4 participate in the response to pressure overload. *Proc.Natl.Acad.Sci.USA.* 1997; **94**:7543-7548.

Hockman, D.J. and Schultz, M.C. Casein kinase II is required for efficient transcription by RNA polymerase III. *Molecular and Cellular Biology.* 1996; **16**:892-898.

Hogan, B.L.M. and Taylor, A. Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature.* 1981; **291**:235-237.

Hopper, A.K. and Phizicky, E.M. tRNA transfers to the limelight. *Genes and Development.* 2003; **17**:162-180.

Hoshijima, M., Minamisawa, S., Yasukawa, H. and Chien, K.R. Molecular pathways for cardiac hypertrophy and heart failure progression. *In Molecular Basis of Cardiovascular Disease, second edition*. Editor: Chien, K.R. Saunders, Philadelphia; 2004: 273-292.

Hsieh, Y.J., Wang, Z., Kovelman, R., and Roeder, R.G. Cloning and characterization of two evolutionary conserved subunits (TFIIIC102 and TFIIIC63) of human TFIIIC and their involvement in functional interactions with TFIIIB and RNA polymerase III. *Molecular and Cellular Biology*. 1999a; **19**:4944-4952.

Hsieh, Y.J., Kundu, T.K., Wang, Z., Kovelman, R., and Roeder, R.G. The TFIIIC90 subunit of TFIIIC interacts with multiple components of the RNA polymerase III machinery and contains a histone-specific acetyltransferase activity. *Molecular and Cellular Biology*. 1999b; **19**:7697-7704.

Hu, P., Wu, S., and Hernandez, N. A minimal RNA polymerase III transcription system from human cells reveals positive and negative regulatory roles for CK2. *Molecular Cell*. 2003; **12**:699-709.

Hu, P., Samudre, K., Wu, S., Sun, Y., and Hernandez, N. CK2 phosphorylation of Bdp1 executes cell cycle-specific RNA polymerase III transcription repression. *Molecular Cell*. 2004; **16**:81-92.

Huang, S. and Houghton, P.J. Targeting mTOR signaling for cancer therapy. *Current Opinion in Pharmacology*. 2003; **3**:371-377.

Huet, J., Conesa, C., Manaud, N., Caussivert, N., and Sentenac, A. Interactions between yeast TFIIIB components. *Nucleic Acids Research*. 1994; **22**:3433-3439.

Hulleman, E. and Boonstra, J. Regulation of G1 phase progression by growth factors and the extracellular matrix. *Cellular and Molecular Life Sciences*. 2001; **58**:80-93.

Hunter, J.J. and Chien, K.R. Signaling pathways for cardiac hypertrophy and failure. *New England Journal of Medicine*. 1999; **341**:1276-1283.

Iijima, Y., Laser, M., Shiraishi, H., Willey, C.D., Sundaravadivel, B., Xu, L., McDermott, P.J., and Kuppuswamy, D. c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. *Journal of Biological Chemistry*. 2002; **277**:23065-23075.

International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature*. 2001; **409**:860-921.

Iritani, B.M. and Eisenman, R.N. c-Myc enhances protein synthesis and cell size during B lymphocyte development. *Proc.Natl.Acad.Sci.USA*. 1999; **96**:13180-13185.

Ito, H., Hirata, Y., Adachi, S., Tanaka, M., Tsujino, M., Koike, A., Nogami, A., Marumo, F., and Hiroe, M. Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes. *Journal of Clinical Investigation*. 1993; **92**:398-403.

Ito, H., Hiroe, M., Hirata, Y., Fujisaki, H., Adachi, S., Akimoto, H., Ohta, Y., and Marumo, F. Endothelin ET_A receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload. *Circulation*. 1994; **89**:2198-2203.

Iwaki, K.I., Sukhatme, V.P., Shubeita, H.E., and Chien, K.R. α- and β-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. *Journal of Biological Chemistry*. 1990; **265**:13809-13817.

Izumo, S., Nadal-Ginard, B., and Mahdavi, V. Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc.Natl.Acad.Sci.USA*. 1988; **85**:339-343.

Jentsch, S. and Schlenker, S. Selective protein degradation: a journey's end within the proteasome. *Cell*. 1995; **82**:881-884.

Johnson, L.F., Abelson, H.T., Green, H., and Penman, S. Changes in RNA in relation to growth of the fibroblast. I. Amounts of mRNA, rRNA, and tRNA in resting and growing cells. *Cell.* 1974; **1**:95-100.

Johnston, G.C., Pringle, J.R., and Hartwell, H. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Experimental Cell Research.* 1977; **105**:79-98.

Johnston, I.M., Allison, S.J., Morton, J.P., Schramm, L., Scott, P.H., and White, R.J. CK2 forms a stable complex with TFIIIB and activates RNA polymerase III transcription in human cells. *Molecular and Cellular Biology.* 2002; **22**:3757-3768.

Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N., and Gallant, P. *Drosophila myc* regulates cellular growth during development. *Cell.* 1999; **98**:779-790.

Jones, T.R. and Cole, M.D. Rapid cytoplasmic turnover of *c-Myc* mRNA: requirement of the 3' untranslated sequences. *Molecular and Cellular Biology.* 1987; **7**:4513-4521.

Kaelin, W.G. Functions of the retinoblastoma protein. *BioEssays.* 1999; **21**:950-958.

Kai, H., Muraishi, A., Sugiu, Y., Nishi, H., Seki, Y., Kuwahara, F., Kimura, A., Kato, H., and Imaizumi, T. Expression of proto-oncogenes and gene mutation of sarcomeric proteins in patients with hypertrophic cardiomyopathy. *Circulation Research.* 1998; **83**:594-601.

Kannel, W.B. Influence of multiple risk factors on the hazard of hypertension. *Journal of Cardiovascular Pharmacology.* 1990; **16 (suppl. 5)**:S53-S57.

Kassavetis, G.A., Bartholomew, B., Blanco, J.A., Johnson, T.E., and Geiduschek, E.P. Two essential components of the *Saccharomyces cerevisiae* transcription factor TFIIIB: transcription and DNA-binding properties. *Proc.Natl.Acad.Sci.USA.* 1991; **88**:7308-7312.

Kassavetis, G.A., Joazeiro, C.A.P., Pisano, M., Geiduschek, E.P., Colbert, T., Hahn, S., and Blanco, J.A. The role of the TATA-binding protein in the assembly and function of the multisubunit yeast RNA polymerase III transcription factor, TFIIIB. *Cell.* 1992; **71**:1055-1064.

Kassavetis, G.A., Kumar, A., Letts, G.A., and Geiduschek, E.P. A post-recruitment function for the RNA polymerase III transcription initiation factor TFIIIB. *Proc.Natl.Acad.Sci.USA.* 1998; **95**:9196-9201.

Kassavetis, G.A., Letts, G.A., and Geiduschek, E.P. The RNA polymerase III transcription initiation factor TFIIIB participates in two steps of promoter opening. *EMBO Journal.* 2001; **20**:2823-2834.

Kelly, K., Cochran, B.H., Stiles, C.D., and Leder, P. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. *Cell.* 1983; **35**:603-609.

Kerkhoff, E. and Rapp, U.R. Cell cycle targets of Ras/Raf signalling. *Oncogene.* 1998; **17**:1457-1462.

Killander, D. and Zetterberg, A. A quantitative cytochemical investigation of the relationship between cell mass and initation of DNA synthesis in mouse fibroblasts *in vitro*. *Experimental Cell Research.* 1965; **40**:12-20.

Kim, S., Li, Q., Dang, C.V., and Lee, L.A. Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc *in vivo*. *Proc.Natl.Acad.Sci.USA.* 2002; **97**:11198-11202.

Kitagawa, M., Higashi, H., Jung, H.K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S., and Taya, Y. The consensus motif for phosphorylation by cyclin D1-cdk4 is different from that for phosphorylation by cyclin A/E-cdk2. *EMBO Journal.* 1996; **15**:7060-7069.

Kitagawa, N., Goto, M., Kurozumi, K., Maruo, S., Fukayama, M., Naoe, T., Yasukawa, M., Hino, K., Suzuki, T., Todo, S., and Takada, K. Epstein-Barr virus-

encoded poly (A)⁻ RNA supports Burkitt's lymphoma growth through interleukin-10 induction. *EMBO Journal*. 2000; **19**:6742-6750.

Knowlton, K.U., Baracchini, E., Ross, R.S., Henderson, S.A., Evans, S.M., Glembotski, C.C., and Chien, K.R. Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during α-adrenergic stimulation of neonatal rat ventricular cells. *Journal of Biological Chemistry*. 1991; **266**:7759-7768.

Knudsen, E.S. and Wang, J.Y.J. Differential regulation of retinoblastoma protein function by specific cdk phosphorylation sites. *Journal of Biological Chemistry*. 1996; **271**:8313-8320.

Koh, K.N., Kang, M.J., Frith-Terhune, A., Park, S.K., Kim, I., Lee, C.O., and Koh, G.Y. Persistant and heterogeneous expression of the cyclin-dependent kinase inhibitor, $p27^{KIP1}$, in rat hearts during development. *Journal of Molecular and Cellular Cardiology*. 1998; **30**:463-474.

Kolodziejczyk, S.M., Wang, L., Balazsi, K., DeRepentigny, Y., Kothary, R., and Megeney, L.A. MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. *Current Biology*. 1999; **9**:1203-1206.

Komano, J., Maruo, S., Kurozumi, K., Oda, T., and Takada, K. Oncogenic role of Epstein-Barr virus-encoded RNAs in Burkitt's lymphoma cell line Akata. *Journal of Virology*. 1999; **73**:9827-9831.

Komuro, I., Kurabayashi, M., Takaku, F., and Yazaki, Y. Expression of cellular oncogenes in the myocardium during the developmental stage and pressure-overloaded hypertrophy of the rat heart. *Circulation Research*. 1988; **62**:1075-1079.

Komuro, I., Kaida, T., Shibazaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F., and Yazaki, Y. Stretching cardiac myocytes stimulates protooncogene expression. *Journal of Biological Chemistry*. 1990; **265**:3595-3598.

Komuro, I., Katoh, Y., Kaida, T., Shibazaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *Journal of Biological Chemistry*. 1991; **266**:1265-1268.

Kovelman, R. and Roeder, R.G. Purification and characterization of two forms of human transcription factor IIIC. *Journal of Biological Chemistry*. 1992; **267**:24446-24456.

Kozar, K., Ciemerych, M.A., Rebel, V.I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R.T., Akashi, K., and Sicinski, P. Mouse development and cell proliferation in the absence of D-cyclins. *Cell*. 2004; **118**:477-491.

Kundu, T.K., Wang, Z., and Roeder, R.G. Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. *Molecular and Cellular Biology*. 1999; **19**:1605-1615.

Kunkel, G.R. and Pederson, T. Upstream elements required for efficient transcription of a human U6 RNA gene resemble those of U1 and U2 genes even though a different polymerase is used. *Genes and Development*. 1988; **2**:196-204.

Kunkel, G.R. and Pederson, T. Transcription of a human U6 small nuclear RNA gene *in vivo* withstands deletion of intragenic sequence but not of an upstream TATATA box. *Nucleic Acids Research*. 1989; **17**:7371-7379.

Kuwahara, K., Saito, Y., Takano, M., Arai, Y., Yasuno, S., Nakagawa, Y., Takahashi, N., Adachi, Y., Takemura, G., Horie, M., Miyamoto, Y., Morisaki, T., Kuratomi, S., Noma, A., Fujiwara, H., Yoshimasa, Y., Kinoshita, H., Kawakami, R., Kishimoto, I., Nakanishi, M., Usami, S., Saito, Y., Harada, M., and Nakao, K. NRSF regulates the fetal cardiac gene program and maintains normal cardiac structure and function. *EMBO Journal*. 2003; **22**:6310-6321.

Lafontaine, D.L.J. and Tollervey, D. The function and synthesis of ribosomes. *Nature Reviews Molecular Cell Biology*. 2001; **2**:514-520.

Lagna, G., Kovelman, R., Sukegawa, J., and Roeder, R.G. Cloning and characterization of an evolutionary divergent DNA-binding subunit of mammalian TFIIIC. *Molecular and Cellular Biology*. 1994; **14**:3053-3064.

Larminie, C.G.C., Cairns, C.A., Mital, M., Martin, K., Kouzarides, T., Jackson, S.P., and White, R.J. Mechanistic analysis of RNA polymerase III regulation by the retinoblastoma protein. *EMBO Journal*. 1997; **16**:2061-2071.

Larminie, C.G.C., Sutcliffe, J.E., Tosh, K., Winter, A.G., Felton-Edkins, Z.A., and White, R.J. Activation of RNA polymerase III transcription in cells transformed by Simian Virus 40. *Molecular and Cellular Biology*. 1999; **19**:4927-4934.

Lavoie, J.N., L'Allemain, G., Brunet, A., Müller, R., and Pouysségur, J. Cyclin D1 expression is regulated positively by the p42/p44^{MAPK} and negatively by the p38/HOG^{MAPK} pathway. *Journal of Biological Chemistry*. 1996; **271**:20608-20616.

Lee, H., Henderson, S., Reynolds, R., Dunnmon, P., Yuan, D., and Chien, K.R. α-1 adrenergic stimulation of cardiac gene transcription in neonatal rat myocardial cells: effects on myosin light chain-2 gene expression. *Journal of Biological Chemistry*. 1988; **263**:7352-7358.

Lee, J.-Y. and Engelke, D.R. Partial characterization of an RNA component that copurifies with *Saccharomyces cerevisiae* RNase P. *Molecular and Cellular Biology*. 1989; **9**:2536-2543.

Lee, J.-Y., Rohlman, C.E., Molony, L.E., and Engelke, D.R. Characterization of *RPR1*, an essential gene encoding the RNA component of *Saccharomyces cerevisiae* nuclear RNase P. *Molecular and Cellular Biology*. 1991; **11**:721-730.

Leresche, A., Wolf, V.J., and Gottesfeld, J.M. Repression of RNA polymerase II and III transcription during M phase of the cell cycle. *Experimental Cell Research.* 1996; **229**:282-288.

Leu, M., Ehler, E., and Perriard, J.C. Characterisation of postnatal growth of the murine heart. *Anatomy and Embryology.* 2001; **204**:217-224.

Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B., and Castelli, W.P. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *New England Journal of Medicine.* 1990; **322**:1561-1566.

Li, T., Spearow, J., Rubin, C.M., and Schmid, C.W. Physiological stresses increase mouse short interspersed element (SINE) RNA expression *in vivo*. *Gene.* 1999; **239**:367-372.

Liang, Q., Wiese, R.J., Bueno, O.F., Dai, Y.F., Markham, B.E., and Molkentin, J.D. The transcription factor GATA4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. *Molecular and Cellular Biology.* 2001; **21**:7460-7469.

Liao, H.J., Kobayashi, R., and Mathews, M.B. Activities of adenovirus-associated RNAs: purification and characterization of RNA binding proteins. *Proc.Natl.Acad.Sci.USA.* 1998; **95**:8514-8519.

Liebhaber, S.A., Wolf, S., and Schlessinger, D. Differences in rRNA metabolism of primary and SV40-transformed human fibroblasts. *Cell.* 1978; **13**:121-127.

Linzbach, A.J. Heart failure from the point of view of quantitative anatomy. *American Journal of Cardiology.* 1960; **5**:370-382.

Liu, W.M., Chu, W.M., Choudary, P.V., and Schmid, C.W. Cell stress and translational inhibitors transiently increase the abundance of mammalian SINE transcripts. *Nucleic Acids Research.* 1995; **23**:1758-1765.

Lobo, S.M. and Hernandez, N. A 7bp mutation converts a human RNA polymerase II snRNA promoter into an RNA polymerase III promoter. *Cell.* 1989; **58**:55-67.

Lobo, S.M., Lister, J., Sullivan, M.L., and Hernandez, M. The cloned RNA polymerase II transcription factor TFIID selects RNA polymerase III to transcribe the human U6 gene in vitro. *Genes and Development.* 1991; **5**:1477-1489.

López-de-León, A., Librizzi, M., Puglia, K., and Willis, I.M. *PCF4* encodes an RNA polymerase III trasncription factor with homology to TFIIB. *Cell.* 1992; **71**:211-220.

Lundberg, A.S. and Weinberg, R.A. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Molecular and Cellular Biology.* 1998; **18**:753-761.

Luyken, J., Hannan, R.D., Cheung, J.Y., and Rothblum, L.I. Regulation of rDNA Transcription During Endothelin-1 Induced hypertrophy of neonatal cardiomyocytes: hyperphosphorylation of upstream binding factor, an rDNA transcription factor. *Circulation Research.* 1996; **78**:354-361.

Lüscher, B. Function and regulation of the transcription factors of the Myc/Max/Mad network. *Gene.* 2001; **277**:1-14.

Malumbres, M., Sotillo, R., Santamaria, D., Galán, J., Cerezo, A., Ortega, S., Dubus, P., and Barbacid, M. Mammalian cells cycle without the D-type cyclin-dependent kinases cdk4 and cdk6. *Cell.* 2004; **118**:493-504.

Mauck, J.C. and Green, H. Regulation of pre-transfer RNA synthesis during transition from resting to growing state. *Cell.* 1974; **3**:171-177.

McDermott, P.J. and Morgan, H.E. Contration modulates the capacity for protein synthesis during growth of neonatal heart cells in culture. *Circulation Research.* 1989; **64**:542-553.

McDermott, P.J., Rothblum, L.I., Smith, S.D., and Morgan, H.E. Accelerated rates of ribosomal RNA synthesis during growth of contracting heart cells in culture. *Journal of Biological Chemistry*. 1989; **264**:18220-18227.

McDermott, P.J., Carl, L.L., Conner, K.J., and Allo, S.N. Transcriptional regulation of ribosomal RNA synthesis during growth of cardiac myocytes in culture. *Journal of Biological Chemistry*. 1991; **266**:4409-4416.

McMullen, J.R., Sherwood, M.C., Tarnavski, O., Zhang, L., Dorfman, A.L., Shioi, T., and Izumo, S. Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. *Circulation*. 2004; **109**:3050-3055.

Medema, R.H., Herrera, R.E., Lam, F., and Weinberg, R.A. Growth suppression by $p16^{ink4}$ requires functional retinoblastoma protein. *Proc.Natl.Acad.Sci.USA*. 1995; **92**:6289-6293.

Meidell, R.S., Sen, A., Henderson, S.A., Slahetka, M.F., and Chien, K.R. Alpha 1-adrenergic stimulation of rat myocardial cells increases protein synthesis. *American Journal of Physiology*. 1986; **251**:H1076-H1084.

Meißner, W., Ahlers, A., and Seifart, K.H. The activity of transcription factor PBP, which binds to the PSE of mammalian U6 genes is regulated during differentiation of F9 cells. *Molecular and Cellular Biology*. 1995; **15**:5888-5897.

Milano, C.A., Dolber, P.C., Rockman, H.A., Bond, R.A., Venable, M.E., Allen, L.F., and Lefkowitz, R.J. Myocardial expression of a constitutively active α_{1B}-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc.Natl.Acad.Sci.USA*. 1994; **91**:10109-10113.

Mittal, V., Cleary, M.A., Herr, W., and Hernandez, N. The Oct-1 POU-specific domain can stimulate small nuclear RNA gene transcription by stabilizing the basal transcription complex $SNAP_c$. *Molecular and Cellular Biology*. 1996; **16**:1955-1965.

Mittal, V. and Hernandez, N. Role for the amino-terminal region of human TBP in U6 snRNA transcription. *Science*. 1997; **275**:1136-1149.

Moir, R.D., Sethy-Coraci, I., Puglia, K., Librizzi, M.D., and Willis, I.M. A tetratricopeptide repeat mutation in yeast transcription factor $IIIC_{131}$ ($TFIIIC_{131}$) factilitates recruitment of TFIIB-related factor $TFIIIB_{70}$. *Molecular and Cellular Biology*. 1997; **17**:7119-7125.

Molkentin, J.D. and Markham, B.E. Myocyte-specific enhancer-binding factor (MEF-2) regulates α-cardiac myosin heavy chain gene expression *in vitro* and *in vivo*. *Journal of Biological Chemistry*. 1993; **268**:19512-19520.

Molkentin, J.D. and Dorn II, G.W. Cytoplasmic signalling pathways that regulate cardiac hypertrophy. *Annual Reviews of Physiology*. 2001; **63**:391-426.

Morgan, H.E., Gordon, E.E., Kira, Y., Chua, B.H.L., Russo, L.A., Peterson, C.J., McDermott, P.J., and Watson, P.A. Biochemical mechanisms of cardiac hypertrophy. *Annual Reviews of Physiology*. 1987; **49**:533-543.

Morgan, H.E. and Baker, K.M. Cardiac hypertrophy: mechanical, neural, and endocrine dependence. *Circulation*. 1991; **83**:13-25.

Morimoto, T., Hasegawa, K., Kaburagi, S., Kakita, T., Wada, H., Yanazume, T., and Sasayama, S. Phosphorylation of GATA-4 is involved in α1-adrenergic agonist-responsive transcription of the endothelin-1 gene in cardiac myocytes. *Journal of Biological Chemistry*. 2000; **275**:13721-13726.

Moss, T. and Stefanovsky, V.Y. At the center of eukaryotic life. *Cell*. 2002; **109**:545-548.

Murphy, D., Brickell, P.M., Latchman, D.S., Willison, K., and Rigby, P.W.J. Transcripts regulated during normal embryonic development and oncogenic transformation share a repetitive element. *Cell*. 1983; **35**:865-871.

Murphy, S., Di Liegro, C., and Melli, M. The *in vitro* transcription of the 7SK gene by RNA polymerase III is dependent only on the presence of an upstream promoter. *Cell*. 1987; **51**:81-87.

Murphy, S., Yoon, J.-B., Gerster, T., and Roeder, R.G. Oct-1 and Oct-2 potentiate functional interactions of a transcription factor with the proximal sequence element of small nuclear RNA genes. *Molecular and Cellular Biology*. 1992; **12**:3247-3261.

Nadruz, W., Kobarg, C.B., Constancio, S.S., Corat, P.D.C., and Franchini, K.G. Load-induced transcriptional activation of *c-jun* in rat myocardium: regulation by myocyte enhancer factor 2. *Circulation Research*. 2003; **92**:243-251.

Nagatomo, Y., Carabello, B.A., Hamawaki, M., Nemoto, S., Matsuo, T., and McDermott, P.J. Translational mechanisms accelerate the rate of protein synthesis during canine pressure-overload hypertrophy. *American Journal of Physiology*. 1999; **277**:H2176-H2184.

Narlikar, J.G., Fan, H.Y., and Kingston, R.E. Cooperation between complexes that regulate chromatin structure and transcription. *Cell*. 2002; **108**:475-487.

Neufeld, T.P., de la Cruz, A.F.A., Johnston, L.A., and Edgar, B.A. Coordination of growth and cell division in the *Drosophila* wing. *Cell*. 1998; **93**:1183-1193.

Nguyen, V.T., Kiss, T., Michels, A.A., and Bensaude, O. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature*. 2001; **414**:322-325.

Nilsen, T.W. The case for an RNA enzyme. *Nature*. 2000; **408**:782-783.

Nozato, T., Ito, H., Watanabe, M., Ono, Y., Adachi, S., Tanaka, H., Hiroe, M., Sunamori, M., and Marumo, F. Overexpression of cdk inhibitor $p16^{INK4a}$ by adenovirus vector inhibits cardiac hypertrophy *in vitro* and *in vivo*: a novel strategy for the gene therapy of cardiac hypertrophy. *Journal of Molecular and Cellular Cardiology*. 2001; **33**:1493-1504.

Nurse, P. Genetic control of cell size at cell division in yeast. *Nature*. 1975; **256**:547-551.

O'Connell, B.C., Cheung, A.F., Simkevich, C.P., Tam, W., Ren, X., Mateyak, M.K., and Sedivy, J.M. A large scale gnetic analysis of c-Myc regulated gene expression patterns. *Journal of Biological Chemistry*. 2003; **278**:12563-12573.

Oettel, S., Hartel, F., Kober, I., Iben, S., and Seifart, K.H. Human transcription factors IIIC2, IIIC1 and a novel component IIIC0 fulfil different aspects of DNA binding to various pol III genes. *Nucleic Acids Research*. 1997; **25**:2440-2447.

Omura, T., Yoshiyama, M., Yoshida, K., Nakamura, Y., Kim, S., Iwao, H., Takeuchi, K., and Yoshikawa, J. Dominant negative mutant of c-Jun inhibits cardiomyocyte hypertrophy induced by endothelin 1 and phenylephrine. *Hypertension*. 2002; **39**:81-86.

Pagano, M. and Jackson, P.K. Wagging the dogma: tissue-specific cell cycle control in the mouse embryo. *Cell*. 2004; **118**:535-538.

Pan, J., Singh, U.S., Takahashi, T., Oka, Y., Palm-Leis, A., Herbelin, B.S., and Baker, K.M. PKC mediates cyclic stretch-induced cardiac hypertrophy through Rho family GTPases and mitogen-activated protein kinases in cardiomyocytes. *Journal of Cellular Physiology*. 2004; published online ahead of print.

Paradis, P., Dali-Youcef, N., Paradis, F.W., Thibault, G., and Nemer, M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodelling. *Proc.Natl.Acad.Sci.USA*. 2000; **97**:931-936.

Paradis, R., MacLellan, W.R., Belaguli, N.S., Schwartz, R.J., and Schneider, M.D. Serum response factor mediates AP-1-dependent induction of the skeletal α-actin promoter in ventricular myocytes. *Journal of Biological Chemistry*. 1996; **271**:10827-10833.

Paule, M.R. and White, R.J. Transcription by RNA polymerases I and III. *Nucleic Acids Research*. 2000; **28**:1283-1298.

Pham, F.H., Cole, S.M., and Clerk, A. Regulation of cardiac myocyte protein synthesis through phosphatidylinositol 3' kinase and protein kinase B. *Advances in Enzyme Regulation*. 2001; **41**:73-86.

Pieler, T., Oei, S.-L., Hamm, J., Engelke, U., and Erdmann, V.A. Functional domains of the *Xenopus laevis* 5S gene promoter. *EMBO Journal*. 1985a; **4**:3751-3756.

Pieler, T., Appel, B., Oei, S.-L., Mentzel, H., and Erdmann, V.A. Point mutational analysis of the *Xenopus laevis* 5S gene promoter. *EMBO Journal*. 1985b; **4**:1847-1853.

Pieler, T., Hamm, J., and Roeder, R.G. The 5S gene internal control region is composed of three distinct sequence elements, organized as two functional domains with variable spacing. *Cell*. 1987; **48**:91-100.

Pluta, K., Lefebvre, O., Martin, N.C., Smagowicz, W.J., Stanford, D.R., Ellis, S.R., Hopper, A.K., Sentenac, A., and Boguta, M. Maf1p, a negative effector of RNA polymerase III in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*. 2001; **21**:5031-5040.

Pombo, A., Jackson, D.A., Hollinshead, M., Wang, Z., Roeder, R.G., and Cook, P.R. Regional specialisation in human nuclei: visualisation of discrete sites of transcription by RNA polymerase III. *EMBO Journal*. 1999; **18**:2241-2253.

Poolman, R.A. and Brooks, G. Expressions and activities of cell cycle regulatory molecules during the transition from myocyte hyperplasia to hypertrophy. *Journal of Molecular and Cellular Cardiology*. 1998; **30**:2121-2135.

Poolman, R.A., Li, J.M., Durand, B., and Brooks, G. Altered expression of cell cycle proteins and prolonged duration of cardiac myocyte hyperplasia in $p27^{KIP1}$ knockout mice. *Circulation Research*. 1999; **85**:117-127.

Poortinga, G., Hannan, K.M., Snelling, H., Walkley, C.R., Jenkins, A., Sharkey, K., Brandenburger, Y., Palatsides, M., Pearson, R.B., McArthur, G.A., and Hannan, R.D. MAD1 and c-MYC regulate the rDNA transcription factor UBF and rRNA synthesis during granulocyte differentiation. *EMBO Journal*. 2004; **23**:3325-3335.

Proud, C.G. Ras, PI3-kinase and mTOR signaling in cardiac hypertrophy. *Cardiovascular Research*. 2004; **63**:403-413.

Rameau, G., Puglia, K., Crowe, A., Sethy, I., and Willis, I. A mutation in the second largest subunit of TFIIIC increases a rate-limiting step in transcription by RNA polymerase III. *Molecular and Cellular Biology*. 1994; **14**:822-830.

Rat Genome Sequencing Project Consortium. Genome sequence of the brown Norway rat yields insights into mammalian evolution. *Nature*. 2004; **428**:493-521.

Reddy, R., Henning, D., Das, G., Harless, M., and Wright, D. The capped U6 small nuclear RNA is transcribed by RNA polymerase III. *Journal of Biological Chemistry*. 1987; **262**:75-81.

Robbins, R.J. and Swain, J.L. c-myc protooncogene modulates cardiac hypertrophic growth in transgenic mice. *American Journal of Physiology*. 1992; **262**:H590-H597.

Rockman, H.A., Ross, R.S., Harris, A.N., Knowlton, K.U., Steinhelper, M.E., Field, L.J., Ross, J.Jr., and Chien, K.R. Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. *Proc.Natl.Acad.Sci.USA*. 1991; **88**:8277-8281.

Rockman, H.A., Wachhorst, S.P., Mao, L., and Ross, J.Jr. ANG II receptor blockade prevents ventricular hypertrophy and ANF gene expression with pressure overload in mice. *American Journal of Physiology*. 1994; **266**:H2468-H2475.

Rosa, M.D., Gottlieb, E., Lerner, M., and Steitz, J.A. Striking similarities are exhibited by two small Epstein-Barr virus-encoded ribonucleic acids and the adenovirus-associated ribonucleic acids VAI and VAII. *Molecular and Cellular Biology*. 1981; **1**:785-796.

Ross, R.S. Molecular and mechanical synergy: cross-talk between integrins and growth factor receptors. *Cardiovascular Research*. 2004; **63**:381-390.

Rønning, Ø.W., Lindmo, T., Pettersen, E.O., and Seglen, P.O. The role of protein accumulation in the cell cycle control of human NHIK 3025 cells. *Journal of Cellular Physiology*. 1981; **109**:411-418.

Rudin, C.M. and Thompson, C.B. Transcriptional activation of short interspersed elements by DNA-damaging agents. *Genes Chromosomes Cancer*. 2001; **30**:64-71.

Ruf, I.K., Rhyne, P.W., Yang, C., Cleveland, J.L., and Sample, J.T. Epstein-Barr virus small RNAs potentiate tumorigenicity of Burkitt lymphoma cells independently of an effect on apoptosis. *Journal of Virology*. 2000; **74**:10223-10228.

Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T.J., and Izumo, S. Molecular characterisation of the stretch-induced adaptation of cultured cardiac cells: an *in vitro* model of load-induced cardiac hypertrophy. *Journal of Biological Chemistry*. 1992; **267**:10551-10560.

Sadoshima, J., Xu, Y., Slayter, H., and Izumo, S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell*. 1993; **75**:977-984.

Sadoshima, J. and Izumo, S. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO Journal*. 1993a; **12**:1681-1692.

Sadoshima, J. and Izumo, S. Molecular characterisation of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT_1 receptor subtype. *Circulation Research*. 1993b; **73**:413-423.

Sadoshima, J., Qiu, J., Morgan, J.P., and Izumo, S. Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine

kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. *Circulation Research*. 1995; **76**:1-15.

Sadoshima, J. and Izumo, S. Rapamycin selectively inhibits angiotensin II-induced increase in protein synthesis in cardiac myocytes in vitro. *Circulation Research*. 1995; **77**:1040-1052.

Sadoshima, J., Aoki, H., and Izumo, S. Angiotensin II and serum differentially regulate expression of cyclins, activity of cyclin-dependent kinases, and phosphorylation of retinoblastoma gene product in neonatal rat cardiac myocytes. *Circulation Research*. 1997; **80**:228-241.

Sadoshima, J. and Izumo, S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annual Reviews of Physiology*. 1997; **59**:551-571.

Saghir, A.N., Tuxworth, W.J., Hagedorn, C.H., and McDermott, P.J. Modifications of eukaryotic initiation factor 4F (eIF4F) in adult cardiocytes by adenoviral gene transfer: differential effects on eIF4F activity and total protein synthesis rates. *Biochemical Journal*. 2001; **356**:557-566.

Sano, M., Abdellatif, M., Oh, H., Xie, M., Bagella, L., Giordano, A., Michael, L.H., DeMayo, F.J., and Schneider, M.D. Activation and function of cyclin T-Cdk9 (positive transcription elongation factor-b) in cardiac muscle cell hypertrophy. *Nature Medicine*. 2002; **8**:166-170.

Schlüter, K.D., Goldberg, Y., Taimor, G., Schäfer, M., and Piper, H.M. Role of phosphatidylinositol 3-kinase activation in the hypertrophic growth of adult ventricular cardiomyocytes. *Cardiovascular Research*. 1998; **40**:174-181.

Schlüter, K.D., Simm, A., Schäfer, M., Taimor, G., and Piper, H.M. Early response kinase and PI 3-kinase activation in adult cardiomyocytes and their role in hypertrophy. *American Journal of Physiology*. 1999; **276**:H1655-H1663.

Schmidt, E.V. The role of *c-myc* in cellular growth control. *Oncogene*. 1999; **18**:2988-2996.

Schneider, M.D., Payne, P.A., Ueno, H., Perryman, M.B., and Roberts, R. Dissociated expression of *c-myc* and a *fos*-related competence gene during cardiac myogenesis. *Molecular and Cellular Biology*. 1986; **6**:4140-4143.

Schramm, L., Pendergrast, P.S., Sun, Y., and Hernandez, N. Different human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters. *Genes and Development*. 2000; **14**:2650-2663.

Schramm, L. and Hernandez, N. Recruitment of RNA polymerase III to its target promoters. *Genes and Development*. 2002; **16**:2593-2620.

Schuhmacher, M., Staege, M.S., Pajic, A., Polack, A., Weidle, U.H., Bornkamm, G.W., Eick, D., and Kohlhuber, F. Control of cell growth by c-Myc in the absence of cell division. *Current Biology*. 1999; **9**:1255-1258.

Scott, P.H., Cairns, C.A., Sutcliffe, J.E., Alzuherri, H.M., McLees, A., Winter, A.G., and White, R.J. Regulation of RNA polymerase III transcription during cell cycle entry. *Journal of Biological Chemistry*. 2001; **276**:1005-1014.

Sears, R., Leone, G., DeGregori, J., and Nevins, J.R. Ras enhances Myc protein stability. *Molecular Cell*. 1999; **3**:169-179.

Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K., and Nevins, J.R. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes and Development*. 2000; **14**:2501-2514.

Segall, J., Matsui, T., and Roeder, R.G. Multiple factors are required for the accurate transcription of purified genes by RNA polymerase III. *Journal of Biological Chemistry*. 1980; **255**:11986-11991.

Selvetella, G., Hirsch, E., Notte, A., Tarone, G., and Lembo, G. Adaptive and maladaptive hypertrophic pathways: points of convergence and divergence. *Cardiovascular Research*. 2004; **63**:373-380.

Sethy, I., Moir, R.D., Librizzi, M., and Willis, I.M. *In Vitro* evidence for growth regulation of tRNA gene transcription in yeast. *Journal of Biological Chemistry.* 1995; **270**:28463-28470.

Shamji, A.F., Nghiem, P., and Schreiber, S.L. Integration of growth factor and nutrient signaling: implications for cancer biology. *Molecular Cell.* 2003; **12**:271-280.

Sharp, S.J., Shaack, J., Cooley, L., Burke, D.J., and Soll, D. Structure and transcription of eukaryotic tRNA genes. *CRC Critical Reviews in Biochemistry.* 1984; **19**:107-144.

Shen, Y., Igo, M., Yalamanchili, P., Berk, A.J., and Dasgupta, A. DNA binding domain and subunit interactions of transcription factor IIIC revealed by dissection with poliovirus 3C protease. *Molecular and Cellular Biology.* 1996; **16**:4163-4171.

Sherr, C.J. Cancer cell cycles. *Science.* 1996; **274**:1672-1677.

Sherr, C.J. and Roberts, J.M. CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes and Development.* 1999; **13**:1501-1512.

Shioi, T., Kang, P.M., Douglas, P.S., Hampe, J., Yballe, C.M., Lawitts, J., Cantley, C.L., and Izumo, S. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO Journal.* 2000; **19**:2537-2548.

Shioi, T., McMullen, J.R., Kang, P.M., Douglas, P.S., Obata, T., Franke, T.F., Cantley, L.C., and Izumo, S. Akt/protein kinase B promotes organ growth in transgenic mice. *Molecular and Cellular Biology.* 2002; **22**:2799-2809.

Shioi, T., McMullen, J.R., Tarnavski, O., Converso, K., Sherwood, M.C., Manning, W.J., and Izumo, S. Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation.* 2003; **107**:1664-1670.

Shubeita, H.E., McDonough, P.M., Harris, A.N., Knowlton, K.U., Glembotski, C.C., Brown, J.H., and Chien, K.R. Endothelin induction of inositol phospholipid

hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. *Journal of Biological Chemistry*. 1990; **265**:20555-20562.

Siehl, D., Chua, B.H.L., Lautensack-Belser, N., and Morgan, H.E. Faster protein and ribosome synthesis in thyroxine-induced hypertrophy of rat heart. *American Journal of Physiology*. 1985; **248** (Cell Physiol. 17):C309-C319.

Simpson, P. and Savion, S. Differentiation of Rat Myocytes in Single Cell Cultures with and without Proliferating Nonmyocardial Cells. *Circulation Research*. 1982; **50**:101-116.

Simpson, P., McGrath, A., and Savion, S. Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and catecholamines. *Circulation Research*. 1982; **51**:787-801.

Simpson, P. Role of proto-oncogenes in myocardial hypertrophy. *American Journal of Cardiology*. 1988; **62**:13G-19G.

Sontheimer, E.J. The spliceosome shows its metal. *Nature Structural Biology*. 2001; **8**:11-13.

Soonpaa, M.H., Kim, K.K., Pajak, L., Franklin, M., and Field, L.J. Cardiomyocyte DNA synthesis and binucleation during murine development. *American Journal of Physiology*. 1996; **271**:H2183-H2189.

Söderlund, H., Pettersson, U., Vennstom, B., Philipson, L., and Mathews, M.B. A new species of virus-coded low molecular weight RNA from cells infected with Adenovirus type 2. *Cell*. 1976; 7:585-593.

Starksen, N.F., Simpson, P., Bishopric, N., Coughlin, S.R., Lee, W.M.F., Escobedo, J.A., and Williams, L.T. Cardiac myocyte hypertrophy is associated with c-*myc* protooncogene expression. *Proc.Natl.Acad.Sci.USA*. 1986; **83**:8348-8350.

Stefanovsky, V.Y., Pelletier, G., Hannan, R.D., Gagnon-Kugler, T., Rothblum, L.I., and Moss, T. An immediate response of ribosomal transcription to growth factor

stimulation in mammals is mediated by ERK phosphorylation of UBF. *Molecular Cell*. 2001; **8**:1063-1073.

Steinberg, T.H., Mathews, D.E., Durbin, R.D., and Burgess, R.R. Tagetitoxin: a new inhibitor of eukaryotic transcription by RNA polymerase III. *Journal of Biological Chemistry*. 1990; **265**:499-505.

Strickland, S., Smith, K.K., and Marotti, K.R. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell*. 1980; **21**:347-355.

Sugden, P.H. and Clerk, A. "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circulation Research*. 1998; **83**:345-352.

Sugden, P.H. Signaling in myocardial hypertrophy: life after calcineurin? *Circulation Research*. 1999; **84**:633-646.

Sugden, P.H. and Clerk, A. Activation of the small GTP-binding protein Ras in the heart by hypertrophic agonists. *Trends in Cardiovascular Medicine*. 2000; **10**:1-8.

Sussman, M.A., McCulloch, A., and Borg, T.K. Dance band on the *Titanic*: biomechanical signaling in cardiac hypertrophy. *Circulation Research*. 2002; **91**:888-898.

Sutcliffe, J.E., Brown, T.R.P., Allison, S.J., Scott, P.H., and White, R.J. Retinoblastoma protein disrupts interactions required for RNA polymerase III transcription. *Molecular and Cellular Biology*. 2000; **20**:9192-9202.

Svensson, C. and Akusjärvi, G. Adenovirus VA RNA_I: a positive regulator of mRNA translation. *Molecular and Cellular Biology*. 1984; **4**:736-742.

Taigen, T., De Windt, L.J., Lim, H.W., and Molkentin, J.D. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. *Proc.Natl.Acad.Sci.USA*. 2000; **97**:1196-1201.

Takeishi, Y., Huang, Q., Abe, J.I., Glassman, M., Che, W., Lee, J.D., Kawakatsu, H., Lawrence, E.G., Hoit, B.D., Berk, B.C., and Walsh, R.A. Src and multiple MAP kinase actiavtion in cardiac hypertrophy and congestive heart failure under chronic pressure-overload: comparison with acute mechanical stretch. *Journal of Molecular and Cellular Cardiology*. 2001; **33**:1637-1648.

Tamamori-Adachi, M., Ito, H., Nobori, K., Hayashida, K., Kawauchi, J., Adachi, S., Ikeda, M.A., and Kitajima, S. Expression of cyclin D1 and CDK4 causes hypertrophic growth of cardiomyocytes in culture: a possible implication for cardiac hypertrophy. *Biochemical and Biophysical Research Communications*. 2002; **296**:274-280.

Tamamori, M., Ito, H., Hiroe, M., Terada, Y., Marumo, F., and Ikeda, M. Essential roles for G1 cyclin-dependent kinase activity in development of cardiomyocyte hypertrophy. *American Journal of Physiology*. 1998; **275**:H2036-H2040.

Thimmappaya, B., Weinberger, C., Schneider, R.J., and Shenk, T. Adenovirus VAI RNA is required for efficient translation of viral mRNA at late times after infection. *Cell*. 1982; **31**:543-551.

Thorburn, A. Ras activity is required for phenylephrine-induced activation of mitogen-activated protein kinase cascade in cardiac muscle cells. *Biochemical and Biophysical Research Communications*. 1994; **205**:1417-1422.

Tower, J. and Sollner-Webb, B. Polymerase III transcription factor B activity is reduced in extracts of growth-restricted cells. *Molecular and Cellular Biology*. 1988; **8**:1001-1005.

Ueyama, T., Kawashima, S., Sakoda, T., Rikitake, Y., Ishida, T., Kawai, M., Yamashita, T., Ishido, S., Hotta, H., and Yokoyama, M. Requirement of activation of the extracellular signal-regulated kinase cascade in myocardial cell hypertrophy. *Journal of Molecular and Cellular Cardiology*. 2000; **32**:947-960.

Upadhya, R., Lee, J., and Willis, I.M. Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. *Molecular Cell.* 2002; **10**:1489-1494.

Vakili, B.A., Okin, P.M., and Devereux, R.B. Prognostic implications of left ventricular hypertrophy. *American Heart Journal.* 2001; **141**:334-341.

Vasseur, M., Condamine, H., and Duprey, P. RNAs containing B2 repeated sequences are transcribed in the early stages of mouse embryogenesis. *EMBO Journal.* 1985; **4**:1749-1753.

von Harsdorf, R., Abdellatif, M., Hauck, L., Mehrhof, F., Wegenka, U., Cardoso, M.C., and Dietz, R. E2F-1 overexpression in cardiomyocytes induces downregulation of $p21^{CIP1}$ and $p27^{KIP1}$ and release of active cyclin-dependent kinases in the presence on insulin-like growth factor I. *Circulation Research.* 1999; **85**:128-136.

Vousden, K.H. p53: death star. *Cell.* 2000; **103**:691-694.

Wada, H., Ivester, C.T., Carabello, B.A., Cooper IV, G., and McDermott, P.J. Translation Initiation Factor eIF-4E. A link between cardiac load and protein synthesis. *Journal of Biological Chemistry.* 1996; **271**:8359-8364.

Wang, H.D., Yuh, C.H., Dang, C.V., and Johnson, D.L. The hepatitis B virus X protein increases the cellular level of TATA-binding protein, which mediates transactivation of RNA polymerase III genes. *Molecular and Cellular Biology.* 1995; **15**:6720-6728.

Wang, L., Gout, I., and Proud, C.G. Cross-talk between the ERK and p70 S6 kinase (S6K) signalling pathways: MEK-dependent activation of S6K2 in cardiomyocytes. *Journal of Biological Chemistry.* 2001; **276**:32670-32677.

Wang, L. and Proud, C.G. Regulation of the phosphorylation of elongation factor 2 by MEK-dependent signalling in adult rat cardiomyocytes. *FEBS Letters.* 2002a; **531**:285-289.

Wang, L. and Proud, C.G. Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes. *Circulation Research*. 2002b; **91**:821-829.

Waspe, L.E., Ordahl, C.P., and Simpson, P.C. The cardiac β-myosin heavy chain isogene is induced selectively in α_1-adrenergic receptor-stimulated hypertrophy of cultured rat heart myocytes. *Journal of Clinical Investigation*. 1990; **85**:1206-1214.

Weber, J.D., Raben, D.M., Phillips, P.J., and Baldassare, J.J. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G_1 phase. *Biochemical Journal*. 1997; **326**:61-68.

Weigmann, K., Cohen, S.M., and Lehner, C.F. Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of *Drosophila* Cdc2 kinase. *Development*. 1997; **124**:3555-3563.

Weinberg, R.A. The retinoblastoma protein and cell cycle control. *Cell*. 1995; **81**:323-330.

Weinmann, R., Raskas, H.J., and Roeder, R.G. Role of DNA-dependent RNA polymerases II and III in transcription of the adenovirus genome late in productive infection. *Proc.Natl.Acad.Sci.USA*. 1974; **71**:3426-3430.

Weissman, A.M. Themes and variations of ubiquitylation. *Nature Reviews Molecular Cell Biology*. 2001; **2**:169-178.

Weser, S., Gruber, C., Hafner, H.M., Teichmann, M., Roeder, R.G., Seifart, K.H., and Meissner, W. Transcription factor (TF)-like nuclear regulator, the 250kDa form of *Homo sapiens* TFIIIB", is an essential component of human TFIIIC1 activity. *Journal of Biological Chemistry*. 2004; **279**:27022-27029.

West, M.J., Stoneley, M., and Willis, A.E. Translational induction of the *c-myc* oncogene via activation of the FRAP/TOR signalling pathway. *Oncogene*. 1998; **17**:769-780.

White, R.J., Stott, D., and Rigby, P.W.J. Regulation of RNA polymerase III transcription in response to F9 embyonal carcinoma stem cell differentiation. *Cell.* 1989; **59**:1081-1092.

White, R.J., Gottlieb, T.M., Downes, C.S., and Jackson, S.P. Mitotic regulation of a TATA-binding-protein-containing complex. *Molecular and Cellular Biology.* 1995a; **15**:1983-1992.

White, R.J., Gottlieb, T.M., Downes, C.S., and Jackson, S.P. Cell cycle regulation of RNA polymerase III transcription. *Molecular and Cellular Biology.* 1995b; **15**:6653-6662.

White, R.J., Trouche, D., Martin, K., Jackson, S.P., and Kouzarides, T. Repression of RNA polymerase III transcription by the retinoblastoma protein. *Nature.* 1996; **382**:88-90.

White, R.J. Transcription Factor IIIB: An important determinant of biosynthetic capacity that is targeted by tumour supressors and transforming proteins (Review). *International Journal of Oncology.* 1998; **12**:741-748.

White, R.J. Control of growth and proliferation by the retinoblastoma protein. *Gene Therapy and Molecular Biology.* 1998; **1**:613-628.

White, R.J. *Gene transcription. Mechanisms and control.* Blackwell Science Ltd., Oxford, U.K. 2001.

White, R.J. *RNA polymerase III transcription, third edition.* Landes Bioscience, Austin. 2002; http://www.eurekah.com.

White, R.J. RNA polymerase III transcription and cancer. *Oncogene.* 2004a; **23**:3208-3216.

White, R.J. RNA polymerase III transcription - a battlegound for tumour suppressors and oncogenes. *European Journal of Cancer.* 2004b; **40**:21-27.

Whitmarsh, A.J. and Davis, R.J. A central control for cell growth. *Nature*. 2000; **403**:255-256.

Wilkinson, M.G. and Millar, J.B. Control of the eukaryotic cell cycle by MAP kinase signaling pathways. *FASEB Journal*. 2000; **14**:2147-2157.

Winter, A.G., Sourvinos, G., Allison, S.J., Tosh, K., Scott, P.H., Spandidos, D.A., and White, R.J. RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumours. *Proc.Natl.Acad.Sci.USA*. 2000; **97**:12619-12624.

Wolffe, A.P. and Brown, D.D. Developmental regulation of two 5S ribosomal RNA genes. *Science*. 1988; **241**:1626-1632.

Wool, I.G. The structure and function of eukaryotic ribosomes. *Annual Reviews of Biochemistry*. 1979; **48**:719-754.

Xiao, G., Mao, S., Baumgarten, G., Serrano, J., Jordan, M.C., Roos, K.P., Fishbein, M.C., and MacLellan, W.R. Inducible activation of c-Myc in adult myocardium in vivo provokes cardiac myocyte hypertrophy and reactivation of DNA synthesis. *Circulation Research*. 2001; **89**:1122-1129.

Yamazaki, T., Komuro, I., and Yazaki, Y. Signalling pathways for cardiac hypertrophy. *Cellular Signalling*. 1998; **10**:693-698.

Yanazume, T., Hasegawa, K., Morimoto, T., Kawamura, T., Wada, H., Matsumori, A., Kawase, Y., Hirai, M., and Kita, T. Cardiac p300 is involved in myocyte growth with decompensated heart failure. *Molecular and Cellular Biology*. 2003; **23**:3593-3606.

Yang, Z., Zhu, Q., Luo, K., and Zhou, Q. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcripion. *Nature*. 2001; **414**:317-322.

Yean, S.-L., Wuenschell, G., Termini, J., and Lin, R.-J. Metal ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome. *Nature*. 2000; **408**:881-884.

Yoon, J.-B., Murphy, S., Bai, L., Wang, Z., and Roeder, R.G. Proximal sequence element-binding transcription factor (PTF) is a multisubunit complex required for transcription of both RNA polymerase II- and RNA polymerase III-dependent small nuclear RNA genes. *Molecular and Cellular Biology*. 1995; **15**:2019-2027.

Yoshinaga, S.K., Boulanger, P.A., and Berk, A.J. Resolution of human transcription factor TFIIIC into two functional components. *Proc.Natl.Acad.Sci.USA*. 1987; **84**:3585-3589.

Yoshinaga, S.K., L'Etoile, N.D., and Berk, A.J. Purification and characterization of transcription factor IIIC2. *Journal of Biological Chemistry*. 1989; **264**:10726-10731.

Yuan, Y. and Reddy, R. 5' flanking sequences of human MRP/7-2 RNA gene are required and sufficient for the transcription by RNA polymerase III. *Biochimica et Biophysica Acta*. 1991; **1089**:33-39.

Yue, T.L., Gu, J.L., Wang, C., Reith, A.D., Lee, J.C., Mirabile, R.C., Kreutz, R., Wang, Y., Maleef, B., Parsons, A.A., and Ohlstein, E.H. Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *Journal of Biological Chemistry*. 2000; **275**:37895-37901.

Zak, R. Development and proliferative capacity of cardiac muscle cells. *Circulation Research*. 1974; **34** and **35** (supplement II):II-17-II-26.

Zaragoza, D., Ghavidel, A., Heitman, J., and Schultz, M.C. Rapamycin induces the G0 program of transcriptional repression in yeast by interfering with the mTOR signaling pathway. *Molecular and Cellular Biology*. 1998; **18**:4463-4470.

Zarkowska, T. and Mittnacht, S. Differential phosphorylation of the retinoblastoma protein by G_1/S cyclin-dependent kinases. *Journal of Biological Chemistry*. 1997; **272**:12738-12746.

Zeller, K.I., Haggerty, T.J., Barrett, J.F., Guo, Q., Wonsey, D.R., and Dang, C.V. Characterization of nucleophosmin (B23) as a Myc target by scanning chromatin immunoprecipitation. *Journal of Biological Chemistry*. 2001; **276**:48285-48291.

Zetterberg, A. and Killander, D. Quantitative cytophotometric and autoradiographic studies on the rate of protein synthesis during interphase in mouse fibroblasts *in vitro*. *Experimental Cell Research*. 1965; **50**:1-11.

Zhang, C.L., McKinsey, T.A., Chang, S., Antos, C.L., Hill, J.A., and Olson, E.N. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell*. 2002; **110**:479-488.

Zhao, J., Yuan, X., Frödin, M., and Grummt, I. ERK-dependent phosphorylation of the transcription initiation factor TIF-1A is required for RNA polymerase I transcription and cell growth. *Molecular Cell*. 2003; **11**:405-413.

Zhou, Z.Q. and Hurlin, P.J. The interplay between Mad and Myc in proliferation and differentiation. *Trends in Cell Biology*. 2001; **11**:S10-S14.

zur Hausen, H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *Journal of the National Cancer Institute*. 2000; **92**:690-698.